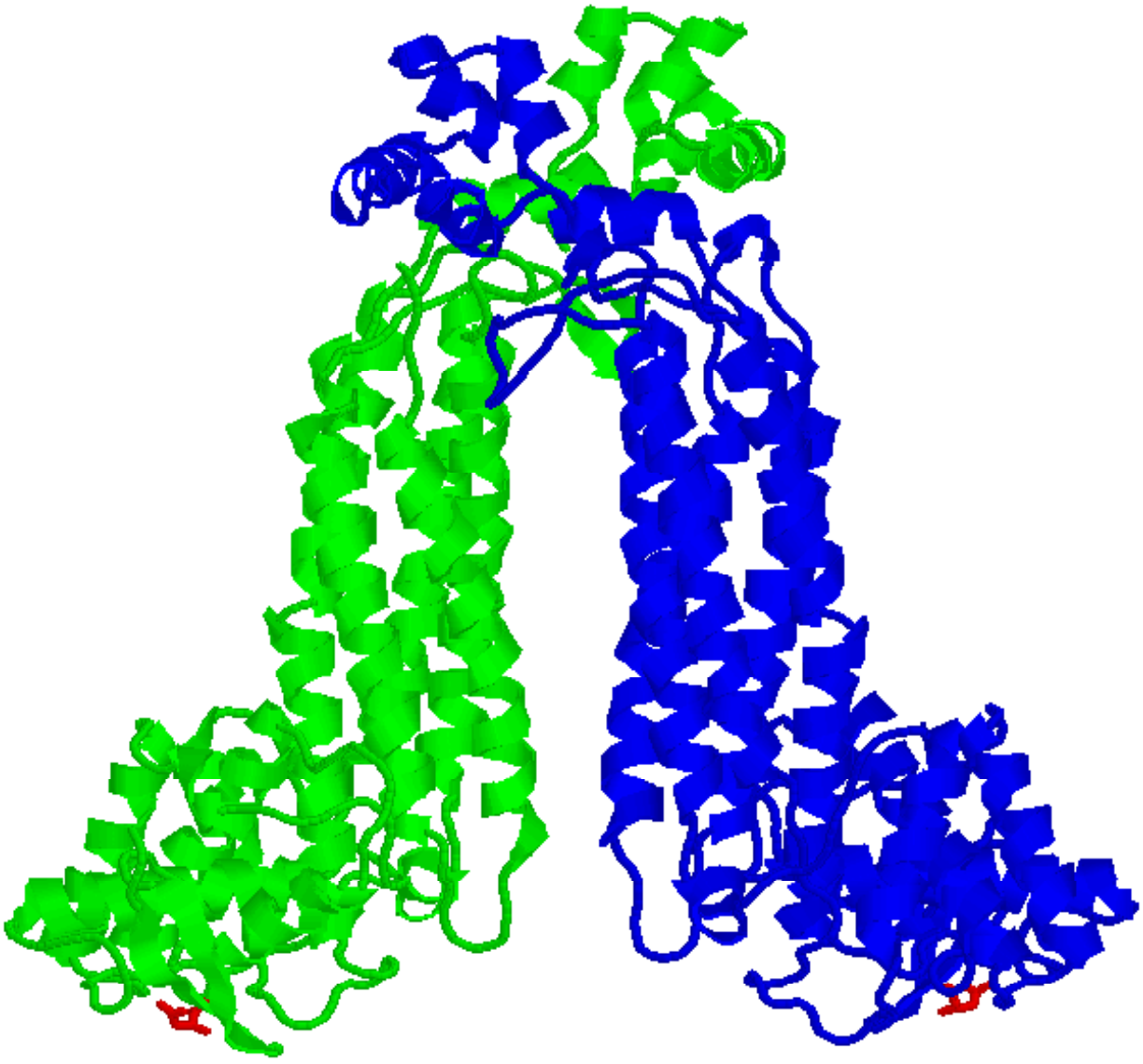
CHEMISTRY 255 — BIOCHEMISTRY

LAB MANUAL



Structure of Fumarate Hydratase with Bound Malate

GUSTAVUS ADOLPHUS COLLEGE

This manual was developed from source
material, used with permission, provided by
Gail Carlson, Ph.D. and Stewart Hendrickson, Ph.D.
at St. Olaf College

Introductory materials were written by Jeff Dahlseid, Ph.D.
Laboratory exercises were developed, adapted,
edited and/or written by Jeff Dahlseid, Ph.D.

CHEMISTRY 255 -- Biochemistry Laboratory -- Fall 2002

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Time and Location: M, T, W or R; 1:30-5:20 p.m in Nobel 207

Laboratory Schedule

Week	Experiment	Techniques Used
9/9	Expt 1: Introduction to basic techniques	Measurements, dilutions, UV/Vis spectroscopy
9/16	Expt 2: Amino acid composition of a dipeptide	Enzymatic proteolysis, paper chromatography
9/23	Discussion on scientific writing; Exercises involving buffer solutions	Read/critique primary literature, Buffer preparation
9/30	———No lab - Nobel Conference——	
10/7	Expt 3: Purification of AMP aminohydrolase	Extract preparation, resin preparation, instrument operation
10/14	Expt 3 (cont'd)	Column chromatography, dialysis, spectroscopic enzyme assay
10/21	———No lab - Reading Break–	**NOTE:** Thursday will meet Oct. 24 instead of Oct. 17
10/28	Expt 3 (cont'd)	Gel electrophoresis, protein concentration and molecular weight determination, enzyme assay
11/4	Expt 4: pH-Dependence of fumarase	Buffer preparation, spectroscopic enzyme assay
11/11	Expt 5: Kinetic analysis of fumarase	Spectroscopic enzyme assay, Michaelis-Menten kinetic analysis
11/18	Individual writing consultation (scheduled)	
11/25	———No lab -Thanksgiving Break——	
12/2	Expt 6: Stereospecificity of the fumarase reaction — an NMR study*	Proton NMR
12/9	To Be Determined	

*Expt. 6 may be done at times scheduled separately for each group.

TABLE OF CONTENTS

Safety Guidelines

* I M P O R T A N T *

POLICY ON SAFETY GOGGLES

ALL STUDENTS WORKING WITH OR IN CLOSE PROXIMITY TO CHEMICALS IN LABORATORIES ARE REQUIRED TO WEAR SAFETY GOGGLES AT ALL TIMES

The chemistry laboratory can be a dangerous place. While little that you will be asked to do is intrinsically dangerous, a majority of the materials that you handle are in some way potentially dangerous if they are handled carelessly. Perhaps the greatest potential danger in your laboratory work will be from corrosive substances such as strong acids and bases that readily attack human tissues.

The most vulnerable part of your body is your eyes and they must be protected. Therefore, we will **INSIST** that approved safety goggles be worn at all times (covering the eyes) when you are working with chemicals or are in the vicinity of others using chemicals. We will not tolerate exceptions to this rule. Your laboratory instructor will remind you to wear safety goggles and you will not be permitted to work in the laboratory if you do not wear them.

Furthermore, at the discretion of the laboratory instructor, should you fail to heed warnings about wearing your safety goggles, you may, after two warnings, be dismissed from the laboratory and required to drop the course. We do not wish to use these penalties. **PLEASE** do not force us to do so. However, be assured that **ALL STUDENTS** will be required to wear safety goggles when working with chemicals and we will not hesitate to assess these penalties to make this requirement work.

If you have a problem with goggles fogging, or if prolonged wear of goggles is uncomfortable, we recommend that you occasionally go into the hallways to remove the goggles for relief; be sure to put them back on prior to returning to the lab. Goggles should **NOT** be removed in the laboratory itself while chemicals are in use. Several models of goggles are available, and we hope that one of these will prove comfortable for you.

In addition, because the safety implications are unclear, it is strongly recommended that contact lenses **NOT** be worn in the laboratory, even under safety goggles. Should chemicals, including solvent vapors, enter the eyes of a student wearing contact lenses, there is a possibility that they chemicals may become trapped under the contact lenses. Damage may be done to the eyes until the lenses can somehow be removed, with the eyesight of the victim being permanently impaired.

Therefore, if you wear contact lenses, you are encouraged to wear glasses instead of contacts in the laboratory. If you wear contacts, but do not own an equivalent pair of glasses, you are encouraged to obtain glasses for laboratory use.

Safety Guidelines

Submission of a Safety Policies and Procedures Signature Sheets (on page after these guidelines and safety information), signed by both the student and laboratory instructor, is required before any student can work in the laboratory.

1. IMMEDIATELY REPORT ANY ACCIDENT OR SAFETY ISSUE TO THE LABORATORY INSTRUCTOR.

Emergency Procedures

2. If chemicals are spilled:

   a. If chemicals are spilled on you or if you see chemicals spilled on another student: Call for help from your instructor. Portions of your body or clothing that have been in contact with the chemicals should immediately be flushed thoroughly with water. Use the safety shower if the spill is extensive. An eye wash is available in the laboratory. Don't wait to see if the chemicals will injure you; rinse spills immediately!

   b. Work with the instructor to clean up spills on the bench or floor. Notify students nearby of the presence of broken glassware and/or chemicals, and keep people from walking through the area until cleanup is complete.

3. In case of fire:

   a. Call for help from your instructor. If clothing is burning slowly walk to the fire blanket and wrap it around yourself, and roll on the floor to extinguish the flames; or slowly walk to the safety shower and pull the cord (or ask someone nearby to pull the cord). [Other students nearby: if necessary, bring the fire blanket to the student or help the student to the shower and pull the chain.]

   b. A fire on the bench top or floor may be put out with a fire extinguisher. Call for help from your instructor.

4. Wash minor cuts or burns with cold water and get band aids or burn ointment from the stockroom. Report all such incidents, no matter how minor, to the instructor.

Safety Goggles and Clothing

5. Safety goggles must be worn at all times in the laboratory. Since under Minnesota law the college is legally responsible for eye accidents if a student is not wearing eye protection, the instructor may dismiss from the laboratory any students not wearing approved goggles. In this class, the instructor will warn students, if necessary, to wear their goggles. If students do not respond to two warnings, the may be dismissed form the laboratory and requested to drop the course. Goggles must be provided by each student and may be purchased through the Gustavus Bookmark.

6. If you are using a computer or printer in a lab, you must wear safety goggles if work with chemicals is occurring in the lab.

7. Clothing: Shoes with solid tops (not sandals) must be worn to protect your feet from broken glass or other sharp materials. Shorts or cut-off pants should not be worn in the lab; it is important to protect your body with clothing rather than risk skin contact with chemicals or other materials. Avoid wearing clothing with loose portions, such as open sweaters, baggy cuffs, and hanging scarves. Loose clothing can catch on glassware and equipment, can drag through spills, and may in some cases be a fire hazard. Similarly, loose, long hair should be tied back so that it does not become entangled in equipment, exposed to chemicals, or provide an impediment to vision.

Handling Chemicals

8. Do not taste or ingest any laboratory chemicals.

9. Avoid touching chemicals with your hands, and always thoroughly wash your hands after using chemicals.

10. Work in a fume hood when carrying out reactions that give off objectionable gases. If you need to smell vapor, do not put your nose directly above a flask, beaker, or other vessel containing chemicals. Holding the vessel at least one foot away, use your hand to gently and very cautiously fan the vapors toward your nose.

11. No mouth pipetting is permitted. When pipetting, use the mechanical pipettors, pipette aids or the rubber bulbs provided in the laboratory.

12. Always add acids to water (its alphabetical!); never add water to acids. Combining acid and water frequently generates heat; addition of the acid to the water reduces the amount of heat generated at the point of mixing and provides more water to disperse the heat.

13. **LABEL** all flasks, beakers, test tubes, and other vessels containing chemicals according to their contents. This facilitates both identifying chemicals during an experiment and following proper waste disposal procedures.

14. Hold reagent bottles and other vessels containing liquids so that any drips will be opposite the label, and hold them so any previous drips on that same side do not get on your hand. Clean off any drips or spills. If necessary, ask for help from your instructor or assistant.

15. Laboratory wastes and residues are to be disposed of in an approved manner. Waste containers for these will be provided, and specific instructions on proper disposal procedures will be provided by the laboratory instructor for each experiment.

Laboratory Supervision

16. No unauthorized experiments are permitted. Students may try new experiments only if they have been discussed with a faculty member and have been approved.

17. No student may work in the laboratory alone. All laboratory work must be under the supervision of a faculty member or student laboratory assistant and at least one other person must be present when work with chemicals is being done. No makeup experiments may be performed without the permission of a student's laboratory instructor.

Behavior in the Laboratory

18. Food and beverages are not permitted in the laboratory. This includes drinking from water bottles that you may carry with you on campus. If you need to eat or drink during the time laboratory is scheduled, you must remove yourself from the laboratory. Be sure to wash your hands before eating after working in laboratory.

19. Backpacks and book bags clutter the benches and/or floors of the laboratory and interrupt traffick flow during laboratory. Please leave your book bags and backpacks in the hallway or another location outside the laboratory.

20. Any "horseplay" or behavior that is harassing, disruptive, or aggressive, or in any way presents a hazard to those in the laboratory is forbidden. Any student or group of students engaging in such behavior may be dismissed by the laboratory instructor and may received no credit for the experiment.

Other Procedures

21. If you anticipate needing to cut glass tubing or insert glass tubing into a rubber stopper, consult with your instructor prior to proceeding. Both procedures present the potential for injury from broken glass, and proper precautions are essential.

22. CLEAN your bench top thoroughly at the end of each laboratory period. This should be done with a damp sponge.

Safe Handling and Disposal of Chemicals

Some information on the safe use and disposal of chemicals is provided in this manual; *additional suggestions will be made by your laboratory instructor*. We want not only to provide you with guidelines about how to use chemicals and equipment safely - no experiment you will do this semester is dangerous if safe procedures are followed throughout - but to be sure that proper precautions are taken to avoid contaminating the environment. To this end, we will provide appropriate containers for disposal of chemical wastes. After collecting these from all the lab sections, our stockroom manager will see to it that proper procedures are observed in disposing of these items.

How does one learn about proper handling of chemicals? We have a variety of sources, the most common of which are Material Safety Data Sheets (MSDS). These provide a variety of information on such things as health hazards, first aid procedures, procedures for cleaning up spills, and disposal procedures for each chemical. An example of an MSDS is copied on pages xi-xv. By law companies that sell chemicals must also provide an MSDS (usually inserted as an extra sheet in shipment) for each chemical shipped. There is no uniform format for an MSDS, so they look very different depending on the source. The Chemistry Department obtains the MSDS forms from the corresponding vendors for the chemicals in the stockroom collection and keeps them on file in the department office. MSDS forms are also available from Sigma-Aldrich over the web. Check out the Chemistry Stockroom link on the Chemistry Department's Home Page or go to www.sigmaaldrich.com/cgi-bin/hsrun/Distributed/HahtShop/HahtShop.htx;start=HS_SeacrchCenter. You may request a copy of an MSDS for one of the compounds you are using if you wish. Or you can download your own from the web.

Codes for Chemical Labeling

In the laboratory you will notice that containers of chemicals are labeled, not only with the name (and in some cases, concentration) of chemicals but also with colored diamonds containing numerical and other codes. These are the NFPA (National Fire Protection Agency) codes and indicate fire, reactivity, health, and other hazards associated with the chemicals. In general, the higher the number of a code, the greater the potential hazard. The specific codes are as follows:

Bottom Diamond: Special Hazards

OXY	Oxidizer
ACID	Acid
ALK	Alkali
COR	Corrosive
W	Use no water
RAD	Radiation

Right Diamond: Reactivity Hazards

0	Stable
1	Unstable if heated
2	Violent chemical change
3	Shock/heat may detonate
4	May detonate

Top Diamond: Fire Hazards

0	Will not burn
1	Burns above 200° F
2	Burns below 200° F
3	Burns below 100° F
4	Burns below 73° F

Left Diamond: Health Hazards

0	Normal material
1	Slightly hazardous
2	Hazardous
3	Extreme danger
4	Deadly

Safe Handling of Biological Material

Biochemists encounter potential laboratory risks in addition to those of other chemical laboratory workers, the risks associated with biological specimens. A problem with these is that there may be risks of which we are not yet aware. However, there are two types of risks we can assess fairly well.

Infectious agents

Agents of infection may be bacterial, viral, or the recently discovered prions (*e.g.* Mad-cow disease).

With any human tissue (including blood), it is now general practice to follow *universal precautions*, handling every sample as if it were infected, because you can never know that it is not. The precautions include persistent use of gloves, segregation of lab from non-lab (*e.g.*, a blood in the lab is never taken out of the lab) *etc.* These are onerous precautions that take a lot of planning and arrangements. Think about, for example, using a phone, looking at your watch or using a personal pen without contamination. Remember that there are viruses, fortunately still rare, that are extremely infectious, more than HIV. In this course we will NOT use human tissue.

Animal tissue may also be contaminated with infectious agents. We will use chicken muscle, and from your kitchen you should know that poultry is a common source of *Salmonella* infections. The problem in the laboratory is that the chicken product may remain infectious long after it is discernable as a chicken product. That is, unlike your kitchen, we do not begin with a cooking process that will kill most microorganisms. Thus, scrupulous cleanliness throughout with personal protection by gloves, taking great care to avoid contacting personal items with the possibility of contaminated gloves, is necessary. Washing hands after lab is highly recommended.

Ecological dangers

In contemporary biochemical laboratories it is common to work with cloning vectors, genetically modified cells and other agents with the potential to modify, mostly in unpredictable ways, major ecological systems. This places a huge burden on the scientist to contain these products, sometimes requiring entire new labs with facilities appropriate to handle the materials. The biggest risk is that it is so easy to forget the possible hazard of something that one works with so routinely. To promote containment, vector-containing material is destroyed through autoclaving. However, in this course we will NOT be working with material involving cloning vectors.

Safety Policies and Procedures Signature Sheet

I have read all the material in the Safety Guidelines section of the Biochemistry Laboratory manual, including the general safety policies, goggles policy and material on safe use and disposal of chemicals. I understand this material and how it applies to the laboratory in which I will be working. I have asked for clarification of those points I do not understand.

Signed: _____ Date: _____

Printed name: _____

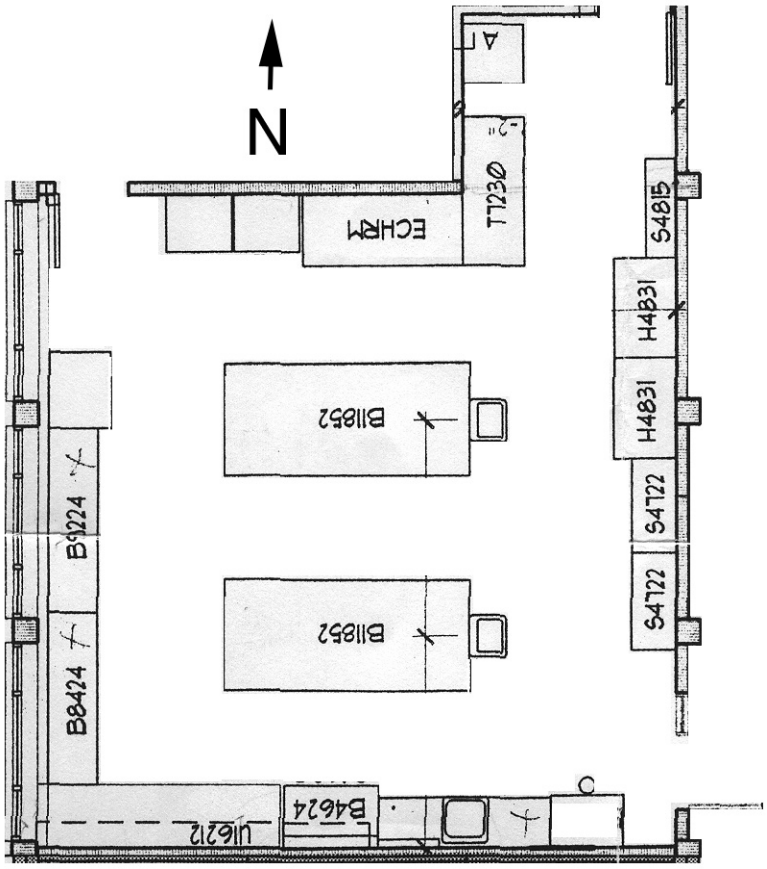
Laboratory section: _____

Accepted by faculty member: _____ Date:_____

Remarks:

Mark the locations of each of the following on the lab

- Eye Wash (1)
- Safety Shower (1)
- Fire extinguisher (1)
- Gas shut off (1)
- Emergency phone (1)
- Exits (2)



Material Safety Data Sheet Example - for ASPIRIN

MSDS Number: A7686 --- Effective Date: 11/17/99

1. Product Identification

Synonyms: 2-(acetyloxy)benzoic acid; salicylic acid acetate; acetysalicylic acid
CAS No.: 50-78-2
Molecular Weight: 180.15
Chemical Formula: C9H8O4
Product Codes: 0033

2. Composition/Information on Ingredients

Ingredient	CAS No	Percent	Hazardous
Acetylsalicylic Acid	50-78-2	100%	Yes

3. Hazards Identification

Emergency Overview

WARNING! HARMFUL IF SWALLOWED OR INHALED. CAUSES IRRITATION TO SKIN, EYES AND RESPIRATORY TRACT. MAY CAUSE ALLERGIC RESPIRATORY REACTION. POSSIBLE BIRTH DEFECT HAZARD. MAY CAUSE BIRTH DEFECTS BASED ON ANIMAL DATA. AFFECTS THE RESPIRATORY SYSTEM,LIVER, KIDNEYS, EYES, SKIN AND BLOOD.

J.T. Baker SAF-T-DATA(tm) Ratings (Provided here for your convenience)

Health Rating: 1 - Slight
Flammability Rating: 1 - Slight
Reactivity Rating: 0 - None
Contact Rating: 1 - Slight
Lab Protective Equip: GOGGLES; LAB COAT
Storage Color Code: Orange (General Storage)

Potential Health Effects

Inhalation:
Causes irritation to the respiratory tract. Exposure may cause an allergy to develop.

Ingestion:
Extremely large oral doses may cause mild burning pain in the mouth and stomach, anorexia, nausea, vomiting, intense thirst, diarrhea, dizziness, irritability, confusion, coma, teratogenic effects, and death from respiratory failure. The mean lethal dose of aspirin by mouth probably lies between 20 and 30 grams in adults.

Skin Contact:
May cause irritation.

Eye Contact:
Causes irritation. Contact cause severe pain and may cause eye damage.

Chronic Exposure:

Repeated ingestion may cause tinitus, abnormal bleeding (gastric or retinal), gastric ulcer, weight loss, mental deterioration, and skin eruptions.

May cause kidney and liver damage in susceptible individuals.

Aggravation of Pre-existing Conditions:

Persons with a history of asthma or allergies to aspirin may be at an increased risk upon exposure to this substance.

4. First Aid Measures

Inhalation:

Remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. Call a physician.

Ingestion:

Induce vomiting immediately as directed by medical personnel. Never give anything by mouth to an unconscious person. Call a physician.

Skin Contact:

In case of contact, immediately flush skin with plenty of soap and water for at least 15 minutes. Remove contaminated clothing and shoes. Wash clothing before reuse. Get medical attention if irritation develops or persists.

Eye Contact:

Immediately flush eyes with plenty of water for at least 15 minutes, lifting lower and upper eyelids occasionally. Get medical attention immediately.

5. Fire Fighting Measures

Fire:

As with most organic solids, fire is possible at elevated temperatures or by contact with an ignition source.

Explosion:

Fine dust dispersed in air in sufficient concentrations, and in the presence of an ignition source is a potential dust explosion hazard. Minimum explosive dust concentration: 40 g/m3.

Fire Extinguishing Media:

Water spray, foam, or dry chemical.

Special Information:

In the event of a fire, wear full protective clothing and NIOSH-approved self-contained breathing apparatus with full face piece operated in the pressure demand or other positive pressure mode.

6. Accidental Release Measures

Remove all sources of ignition. Ventilate area of leak or spill. Wear appropriate personal protective equipment as specified in Section 8.

Spills:

Clean up spills in a manner that does not disperse dust into the air. Use non-sparking tools and equipment. Reduce airborne dust and prevent scattering by moistening with water. Pick up spill for recovery or disposal and place in a closed container.

7. Handling and Storage

Keep in a tightly closed container, stored in a cool, dry, ventilated area. Protect against physical damage. Isolate from any source of heat or ignition. Isolate from incompatible substances. Containers of this material may be hazardous when empty since they retain product residues (dust, solids); observe all warnings and precautions listed for the product.

8. Exposure Controls/Personal Protection

Airborne Exposure Limits:

- ACGIH Threshold Limit Value (TLV): 5 mg/m3 (TWA)

Ventilation System:

A system of local and/or general exhaust is recommended to keep employee exposures below the Airborne Exposure Limits. Local exhaust ventilation is generally preferred because it can control the emissions of the contaminant at its source, preventing dispersion of it into the general work area. Please refer to the ACGIH document, Industrial Ventilation, A Manual of Recommended Practices, most recent edition, for details.

Personal Respirators (NIOSH Approved):

If the exposure limit is exceeded, a half-face dust/mist respirator may be worn for up to ten times the exposure limit or the maximum use concentration specified by the appropriate regulatory agency or respirator supplier, whichever is lowest. A full-face piece dust/mist respirator may be worn up to 50 times the exposure limit, or the maximum use concentration specified by the appropriate regulatory agency, or respirator supplier, whichever is lowest. For emergencies or instances where the exposure levels are not known, use a full-facepiece positive-pressure, air-supplied respirator. WARNING: Air-purifying respirators do not protect workers in oxygen-deficient atmospheres.

Skin Protection:

Wear protective gloves and clean body-covering clothing.

Eye Protection:

Use chemical safety goggles. Maintain eye wash fountain and quick-drench facilities in work area.

9. Physical and Chemical Properties

Appearance: Transparent, colorless crystals.
Odor: Odorless.
Solubility: 1g/100g water @ 37C.
Specific Gravity: 1.40
pH: No information found.
% Volatiles by volume @ 21C (70F): 0
Boiling Point: 140C (284F)
Melting Point: 135C (275F)
Vapor Density (Air=1): No information found.
Vapor Pressure (mm Hg): No information found.
Evaporation Rate (BuAc=1): No information found.

10. Stability and Reactivity

Stability:

Stable in dry air.

Hazardous Decomposition Products:

Decomposes to acetic acid and salicylic acids in the presence of moist air. Carbon dioxide and carbon monoxide may form when heated to decomposition.

Hazardous Polymerization:

Will not occur.

Incompatibilities:

Strong oxidizers, alkali hydroxides, boiling water, and antipyrine, aminopyrine, methamine, phenol, phenyl salicylate, and sodium bicarbonate.

Conditions to Avoid:

Moisture.

11. Toxicological Information

Toxicological Data:

Oral rat LD50: 200 mg/kg; investigated as a mutagen, reproductive effector.

Reproductive Toxicity:

Reproductive effects recorded on humans.

(RTECS)

--------\Cancer Lists\----------

Ingredient	---NTP Carcinogen--- Known	Anticipated	IARC Category
Acetylsalicylic Acid (50-78-2)	No	No	None

12. Ecological Information

Environmental Fate:

When released into the soil, this material may leach into groundwater. When released into the soil, this material may biodegrade to a moderate extent. When released into water, this material is not expected to evaporate significantly. This material has an estimated bioconcentration factor (BCF) of less than 100. This material is not expected to significantly bioaccumulate. When released into the air, this material may be moderately degraded by reaction with photochemically produced hydroxyl radicals. When released into the air, this material may be removed from the atmosphere to a moderate extent by wet deposition.

Environmental Toxicity:

No information found.

13. Disposal Considerations

Whatever cannot be saved for recovery or recycling should be managed in an appropriate and approved waste disposal facility. Processing, use or contamination of this product may change the waste management options. State and local disposal regulations may differ from federal disposal regulations. Dispose of container and unused contents in accordance with federal, state and local requirements.

14. Transport Information

Not regulated.

15. Regulatory Information

--------\Chemical Inventory Status - Part 1\----------

Ingredient	TSCA	EC	Japan	Australia
Acetylsalicylic Acid (50-78-2)	Yes	Yes	Yes	Yes

--------\Chemical Inventory Status - Part 2\----------

Ingredient	Korea	--Canada-- DSL	NDSL	Phil.
Acetylsalicylic Acid (50-78-2)	Yes	Yes	No	Yes

--------\Federal, State & International Regulations - Part 1\----------

Ingredient	-SARA 302- RQ	TPQ	------SARA 313------ List	Chemical Catg.
Acetylsalicylic Acid (50-78-2)	No	No	No	No

--------\Federal, State & International Regulations - Part 2\----------

Ingredient	-RCRA- CERCLA	261.33	-TSCA- 8(d)
Acetylsalicylic Acid (50-78-2)	No	No	No

Chemical Weapons Convention: No TSCA 12(b): No CDTA: No
SARA 311/312: Acute: Yes Chronic: Yes Fire: No Pressure: No
Reactivity: No (Pure / Solid)

WARNING:
THIS PRODUCT CONTAINS A CHEMICAL(S) KNOWN TO THE STATE OF CALIFORNIA TO CAUSE BIRTH DEFECTS OR OTHER REPRODUCTIVE HARM.

Australian Hazchem Code: No information found.
Poison Schedule: S2
WHMIS:
This MSDS has been prepared according to the hazard criteria of the Controlled Products Regulations (CPR) and the MSDS contains all of the information required by the CPR.

16. Other Information

NFPA Ratings: Health: 2 Flammability: 1 Reactivity: 0
Label Hazard Warning:
WARNING! HARMFUL IF SWALLOWED OR INHALED. CAUSES IRRITATION TO SKIN, EYES AND RESPIRATORY TRACT. MAY CAUSE ALLERGIC RESPIRATORY REACTION. POSSIBLE BIRTH DEFECT HAZARD. MAY CAUSE BIRTH DEFECTS BASED ON ANIMAL DATA. AFFECTS THE RESPIRATORY SYSTEM, LIVER, KIDNEYS, EYES, SKIN AND BLOOD.

Label Precautions:
Do not breathe dust.
Avoid contact with eyes, skin and clothing.
Keep container closed.
Use only with adequate ventilation.
Wash thoroughly after handling.
Label First Aid:

If swallowed, induce vomiting immediately as directed by medical personnel. Never give anything by mouth to an unconscious person. If inhaled, remove to fresh air. If not breathing, give artificial respiration. If breathing is difficult, give oxygen. In case of contact, immediately flush eyes or skin with plenty of water for at least 15 minutes. Remove contaminated clothing and shoes. Wash clothing before reuse. In all cases call a physician.

Product Use:
Laboratory Reagent.
Revision Information:
No changes.

CHE 255 Biochemistry Laboratory Fall 2002
Overview and Integrated Schedule

Course Description and Objectives: Biochemistry is the study of the molecules of living systems, including study of the physical structures and interactions, chemical reactions, and biological activities of these molecules. The four main marcomolecules central to biochemical study are proteins, nucleic acids, lipids, and carbohydrates. Through focused study of protein enzymes and their activities, properties, and kinetics, this laboratory course will introduce you to techniques fundamental to biochemical investigations. For this course, my specific objectives are that you:

- understand and use many of the techniques and tools of biochemistry
- comprehend fundamental principles of biochemical research
- communicate research results effectively in written format

Course Activities: As a primary basis for learning, we will use hands-on laboratory work (including keeping a notebook). In addition, you will write short reports, participate in discussion, write an initial and a final draft of a formal laboratory report, and complete data analysis/question reports. An integrated schedule of these activities is shown below; assignments are due on the weekday of your lab section for the week indicated and must be turned in by 5:30 p.m. Detailed descriptions of these projects can be found on page 5 and following. See the CH 255 course syllabus for assessment and grading information and policies.

Biochemistry Laboratory Schedule:

Week of:	Topic
9/9	Expt 1: Introduction to Basic Techniques
9/16	Expt 2: Amino Acid Composition of a Dipeptide *Short report for Expt 1 due*
9/23	Writing discussion and workshop; Buffer preparation *Questions for discussion and buffer problems due*
9/30	Nobel Conference *Short report for Expt 2 due, summary of a Nobel lecture due Oct. 3*
10/7	Expt 3: Purification of AMP Aminohyrolase
10/14	Expt 3: (cont'd)
10/21	Reading Break
10/28	Expt 3: (cont'd)
11/4	Expt 4: pH-Dependence of Fumarase *Expt 4 Data analysis and questions (DAQs)* *Expt 3 formal report initial draft due*
11/11	Expt 5: Kinetic Analysis of Fumarase *Expt 5 DAQs due*
11/18	Writing consultation (scheduled)
11/25	Thanksgiving Break *Final draft of Expt 3 formal report due*
12/2	Expt 6: Stereospecificity of the Fumarase Reaction *Expt 6 DAQs due*
12/9	To be determined

Class policies, structure, e-mail, and feedback: By its very nature, laboratory involves learning through hands-on participation, so you **must** attend laboratory. Failure to do so will result in failure of the laboratory portion of the course (see CHE 255 syllabus). Approval of excuses for missing lab will be determined on a case by case basis. Should you anticipate an acceptable absence, you must let me know ahead of time. This will facilitate making advanced arrangements for acceptable absences.

For each lab we will have a pre-lab discussion to go over background material for the lab, anticipate scheduling and trouble-shooting issues, and communicate new and helpful information. I **strongly** recommend that you prepare for pre-laboratory ahead of time. Doing this will allow you to ask questions about matters you find confusing, which may save you serious time and frustration during the laboratory. Furthermore, by reading the material in the laboratory manual, developing a plan, doing any necessary calculations, and noting questions in advance of lab, you can insure things go more smoothly for you and your group in lab. In addition, the more organized you are when you come to lab, the better chance you will have to finish in reasonable time. To gain as much as possible from laboratory, I recommend that you read any material from the Lehninger text or library reserve papers that are related to the week's exercise.

Presenting scientific results is an essential part of the scientific endeavor, and thus a focus of this class will include scientific writing. Having an accurate record of what you actually did and how it worked is critical to communicating your results. Therefore, you should also keep a notebook during lab of all the information necessary to communicate your results. Keeping this in good form and organized will make reports easier. Descriptions and instructions for written reports, related exercises and the presentation project can be found on page 3 and following.

Science as practiced is increasingly a collaborative endeavor. It is my hope that an environment of active learning and cooperation can be cultivated in laboratory, and I welcome and encourage your participation. Laboratory work is rigorous and I expect everyone to work hard, but if you are having problems with the course please let me know. I am here to help you learn. To help answer questions you have about the course, I will be using e-mail. Expanded comments about e-mail are available upon request. As an additional note, you should be aware that you may use the lab section e-mail alias (f-che-255-#-lab@gac.edu, where # is your lab section number) to ask questions of other lab members in your section.

I expect to be learning a great deal this semester. I am very enthusiastic about being and becoming an excellent educator, both for you and my future students. I welcome your constructive suggestions as to how to improve laboratory and my teaching. I invite you to present and discuss your suggestions with me in my office at any time during the semester.

Note about academic honesty: Issues of academic honesty are a serious matter. As your writing will be a principle vehicle for assessment of your work in the laboratory, it is important that you understand my academic honesty policy, specifically regarding plagiarism. Consult the CHE 255 course syllabus for details about these matters.

Students with disabilities: Appropriate accommodations will be made for students with documented disabilities. If you have a disability requiring accommodation in this class, please contact either me at my extension or Jane Patchin at x7027. Information concerning a disability will be kept strictly confidential.

GUIDELINES FOR WRITING LAB REPORTS

You will be writing short reports (see page 5) for Expt. 1 and 2, and a formal lab report (see page 6) for Expt. 3. You should write your lab reports, short or formal, following the relevant guidelines for the format of a scientific research paper outlined below (NOTE: for short reports only the Materials and Methods and Results sections apply). In addition, you should write your reports for a *peer audience*, specifically so that they can be read and understood by someone with about the same approximate general knowledge of biochemistry. *Keep in mind that scientific writing is concise and terse. Say only what is needed for clarity.* My hope is that by defining your audience for you, it will make easier decisions about what detail should and should not be included. Although I encourage you to work with your lab group to understand the experiments and data, **lab reports are to be written on an individual basis and in your own words.** *Reports must be turned in by 5:30 p.m.the day they are due as indicated on the schedule.*

There are many good reference manuals for scientific writing, including The ACS Style Guide: A Manual for Authors and Editors, edited by Janet S. Dodd (Amer. Chem. Soc.), available in the college library. See also material from J.R.L. Walker on reserve in the library. However, we will also be spending laboratory class time discussing and evaluating samples of scientific writing. There will also be instructor and peer feedback on the first draft of your formal report, followed by a consultation with the instructor, before the final draft is to be compete.

In your reports include the following sections in the order given:

Title page. First page has the paper title, date, author(s) and relevant biographical information.

Abstract. Summarize the major points of the report, including a one sentence statement of what was done/attempted, **all** significant data/results obtained, and a brief statement about the meaning of the results. This section should be very concise and not exceed one paragraph in length.

Introduction. Provide background material needed to support and understand the data presented in the report *from the angle you present it*. This means that your choice about whether to present the material as a novel discovery or as a methods development should impact the content of your introduction. Think about the following questions: What were you trying to accomplish in this experiment? Given your answer to that question, what does the reader need to know or to remember in preparation for reading a description your experimental results? Include in this section an introduction and pertinent background to the topic of the paper, the purpose of your experiment(s), the theoretical basis or rationale for the experiment(s), and the significance (expected or real) of the result(s) you sought. *Do not include any methods or results here.*

Materials and Methods (Experimental). Describe exactly all the methods and reagents ***you used*** in the experiment using passive voice. Where the lab manual or other sources provide method details, I want you to reference the source (with a parenthetical inset) but to write **in your own words** a description of the method. **Be sure** to list your source reference in the bibliography. Copying from the lab manual is not permitted. This section should be as brief as possible, yet allow your *peers* to repeat *exactly* the experiment you described given only your report as a guide. For example, it is important to list the composition of all buffers and other solutions used in the experiment, but not to describe *how* they were prepared or *how much* was made. In contrast, *the amount used* in the particular experiment *may* be important.

Results. **Report all data obtained**. Relevant data should be *clearly* presented in **Tables** and/or as **Figures**. To be clear, all figures and tables must have a *title* (following the figure or

table number in the legend), *properly labeled components (axes, units of measure, etc.),* and include *figure legends* with relevant information not already in the figure such as the identity of symbols used, wavelengths, amount of sample used, etc. *Include only essential information in the figure legends that is already described in the Materials and Methods section*. The Results section **must** also include textual explanations of the results as well. It is often helpful to think of the textual part of the results section as serving the function of ***telling a story*** about your results. This has several implications. First, introductory or transition sentences can be very helpful. The use of these seemingly extra words does not have to be in conflict with being concise, and it may yield clarity that allows it all to be said with less. Secondly, a good story does not cover everything but will report the most significant details. Thus, although a good Results **section** reports all the details, the **text** of your Results may leave some details present only in the tables or figures. The idea is to lead your reader through the results you obtained from your experiments, *not* to simply reiterate the methods. Primary data (spectra and kinetic recordings) should be retained, dated, and kept in your notebooks. It is important to provide equations used to calculate important experimental values, but do not show the actual calculation unless the actual result comes from a computational approach. These can be placed in the methods when VERY routine, in the Results text or Figure Legends, but not more than one of these. I require you to use the past tense in your Results section (what DID you obtain in this experiment?).

Discussion. Analyze the data reported in the results section. Try to answer the questions: What do these data mean? How do the results advance the knowledge in this field of study? What future experiments are suggested by your work? Do not simply summarize the data again. Use the past tense here (what DID this experiment reveal?). *Be sure to address all the questions listed at the end of **each** experiment **in this manual**.*

References. As laboratory research relies on previous work, written reports **must** include references. Inadequate or inappropriate referencing should be avoided. Include references to any journal articles, books, laboratory techniques manuals, etc. used to complete your lab reports **both** as *parenthetical references* (in the correct location of the text) AND in the *bibliography*. List references in proper scientific format. Research journals in biochemistry use varied systems for reference citation. For this course, use the following format:

> First-last-name, First-initial., Second-last-name, Initial., and Last-last-name, Initial. (Date) Title. *Journal Title Standard Abbreviation* **Volume number**, first page-last page.

For further information about writing lab reports, consult the article on reserve in the college library: Walker, J.R.L. (1991) A student's guide to practical write-ups. *Biochem. Ed.* **19**, 31-32.

Lab Notebooks. Presenting scientific results (written or oral) is an essential part of the scientific endeavor, and thus a focus of this class. Having an accurate and real record of what you actually did and what resulted is critical to communicating your results. Therefore, you **must** keep a notebook during the lab that includes all of the information, methods, data, etc. that are necessary to repeat and communicate your results. Researchers in biochemistry use a variety of notebook formats, ranging from bound carbon-copy notebooks to loose paper kept in a ring binder. You may choose your own approach, but your notes **must** be legible, clear, complete, and well-organized so that someone could read, understand, and repeat your work. Ask if you want help with this. It will be **incredibly helpful** to you in creating well-written lab reports.

Other Notes:
You will need a PC-formatted disk on which to save all data generated in this lab, including spectroscopic data. *Nothing will be safe stored only on the hard drive of the computers in lab*.

Short Reports for Laboratory

Project Overview:
These assignments are short length reports, formal in format, and intended to improve your scientific communicating and writing skills by practicing communicating what you did in laboratory to a peer level audience.

Learning Objectives:
- Convey concisely, precisely, and with understanding the methods for the relevant experiment (NOT simply a copy of the lab manual!) in a Materials and Methods section.
- Report in narrative form and with understanding the results of the relevant experiment, supported by all of the data in either tabular or figure format.
- Communicate a well understood and well written report (i.e. complete, concise, and good use of bibliographic references and proper English)

Directions and Format:
The short format report should be a brief, typed report including **only** the Methods and Materials section and Results section (including data analysis and equations) of a formal report plus the answers to questions from DATA ANALYSIS of the experiment. Refer to the preceding pages for direction on the writing of the short report. Additional reference material is available in the library as noted in the lab manual.

A formal length requirement is not going to be enforced, but make sure you follow closely the directions regarding clarity of communication while being concise in your presentation of what you need to say. Format requirements include a report that is typed double spaced, with one inch margins all around and using a font that is no less than 12 points. Placement of figures and tables can be in the text or at the end, but **must have titles and figure legends**. Use headings to identify the Materials and Methods (one section) and Results sections. Note the directions on use of **VERB TENSE** in scientific writing. The report should be nicely printed, but need not be bound except by staple or paper clip.

Evaluation Criteria:
The report will be evaluated based on how well you accomplish the learning objectives outlined above. I want to be convinced from your report that I could repeat the experiment given what you have written. I will also be looking for whether you have done a high quality and complete job in effectively communicating your results, including your ability to write a good narrative, and not just a list of references to the figures and tables. I will be looking for clear and concise communication, completeness, and a good understanding of your topic. I will also be looking for correct labeling of figures (units, labeled axis, etc.) and proper and adequate use of references.

Formal Report for Laboratory

Project Overview:
This assignment is a formal laboratory report to be written in science journal article format. The intent of this project is to provide an opportunity for you to; 1) develop an appreciation for the development and theory of techniques you used in the laboratory exercise, 2) gain a deeper knowledge and understanding of the biochemistry central to the laboratory exercise and the fundamentals of biochemical research in general, and 3) learn how to communicate effectively the results of your laboratory efforts in a professional format. Together, this assignment serves to provide a vehicle toward achieving the course objectives.

The Expt 3 formal report will be initially evaluated (and graded) as a first draft. The writing in the first draft will also be critiqued through a peer review exercise (see below). Two of your peers will critique your draft (and you will critique others' drafts). Peer critiques will be graded! I will assemble the critiques as a whole and provide you with the respective feedback. You will then have the opportunity to discuss your writing with me during a scheduled consultation. The final draft of the Expt 3 formal report will be due one week later. (see syllabus page 1 for dates)

Learning Objectives:
- Convey an understanding of the techniques (use, theory, etc.) used in the laboratory exercise in the context of presenting your results
- Demonstrate knowledge and understanding of the biochemistry and the approach of the exercise and appreciate the significance of your results
- Communicate a well researched and well written report (i.e. complete, concise, and good use of bibliographic references and proper English)

Directions and Format:
The directions for writing formal laboratory reports are well elaborated on pages 3-4. Additional reference material is available in the library as noted. A formal length requirement will not be enforced, but make sure you follow closely the directions regarding clarity of communication while being concise in your presentation of what you need to say. Format requirements include a report that is typed double-spaced, with one inch margins all around and using a font that is no less than 12 points. Placement of figures and tables can be in the text or at the end, but **figure legends must accompany them**. Headings should be used for each of the key sections of a scientific article, including abstract, introduction, materials and methods (one section), results, discussion, and references. Note the directions on use of **VERB TENSE** in scientific writing. The report should be a quality printing, but need not be bound except by staple or paper clip.

Evaluation Criteria:
The report will be evaluated based on how well you accomplish the learning objectives outlined above. I want to be convinced from your report that you have done a high quality and complete job in effectively communicating your results. In your writing I will be looking for clear and concise communication, completeness, and a good understanding of your topic. I will also be looking for correct labeling of figures (units, labeled axis, etc.), proper and adequate use of references, and a discussion that is thoughtful and possibly even creative.

Scientific Writing Discussion

For this exercise, we will be discussing and hopefully learning about scientific writing. The vehicle for our discussion will be in part your reflection and responses to reading the writing in the two successfully published biochemical papers provided. We will be analyzing the papers for their writing, rather than their scientific merit. Learning to think about the intended reader before or while writing is a great vehicle for clear writing. However, to aid in your assessment of the writing you will need to have a sense for the science presented. I recommend reading both papers once to get a general sense for their content, the types of experimental questions asked, the types of experimental techniques employed, and the outcome and significance of the work. After you have done this, you should read the list of questions or points for reflection below and then re-read and analyze the writing in both papers. The papers I have provided intentionally represent two different formats so that comparison and contrast might aid our discussion. The main idea behind this exercise is that reflecting upon and discussing scientific writing as a reader should make more explicit to you elements of scientific writing that make it good. Once you recognize these elements explicitly, you can then apply yourself to emulating those elements of quality in your own writing

How does the writing in these papers differ from other types of writing you have encountered? How does the writing in the two papers differ from each other?

What is the general scheme for organization of both papers? How do they differ?

Look at the respective sections of each paper in light of the function outlined for them in the laboratory manual (pp. 3-4). How do the sections of each paper fall short, meet, or exceed these goals/functions? How are the papers similar or different in this regard?

In order for others to repeat published work, it is very important that the description of what was done is complete. Could you repeat the experiments (or does it seem like you could) described in either paper given what the authors provide/describe? Is one of the papers better in this regard? Which one and why?

Figures and tables and their legends play a key role in effectively and clearly presenting scientific results. Can you tell what question the experiment in the figure is trying to answer? Can you tell what techniques were used to obtain the results in the figure? How were the samples (if any) prepared? How do the symbols or labels aid your understanding of the data? What features are most helpful or essential?

What do you notice about the verb tense used throughout the different sections of a given paper? Within the individual sections of a given paper? Between the two different papers? What role does this play?

Clarity of scientific writing is of key importance. Are the respective sections of each paper clear? Are the figures, tables and figure legends (as presented) clear? Overall, what do you think contributes to whether these papers achieve clarity (or fail to!)? For example, how does their organization, format, use of verb tense, etc. help or hinder this? Is one paper more clear than the other? If so, why?

Having thoughtfully analyzed these papers as a reader, what would be your main goal (i.e. what would you try to achieve) as you write a scientific paper? What would you try and avoid?

What other observations or questions do you have concerning the writing in these two paper?

EXPERIMENT 1. Introduction to Basic Biochemical Techniques

Biochemistry is a very broad discipline, but there are certain basic techniques that every biochemist uses in the laboratory. Three of these basic techniques are emphasized in this exercise and they are: **(1) Measurements, (2) Dilutions, and (3) UV/vis Spectroscopy**. This experiment introduces you to these essential techniques and gives you a chance to try them for yourself. In this experiment you will utilize these techniques to measure the concentration of protein in solution (the Bradford assay), although you will use these techniques repeatedly throughout the semester.

(1) MEASUREMENTS

Many biochemical measurements are made on a very small scale using micropipettors, many of which are air displacement piston pipettes, such as the Rainin Pipetman™. They are quick and convenient, but their accuracy is dependent on how they are adjusted and used. Below are volume ranges, cautions, and instructions for using the pipettors. If you have not used pipettors before, or if it has been a while, practice using them with water before proceeding to the Bradford assay.

Volume Ranges for Pipettors: In biochemistry laboratory, we will use sets of pipettors, which measure volumes on a microliter scale. The following summarizes the three basic models. **Small-volume model**: generally 1-20 µl in 0.02 µl increments (recommended range is 1-20 µl). **Mid-volume model**: 1-200 µl in 0.2 µl increments (recommended range is 20-200 µl). **Large-volume model**: 1-1,000 µl in 2.0 µl increments (recommended range is 200-1,000 µl).

CAUTIONS for using the pipettors:

1. **NEVER** invert the pipettor or lay it on its side with liquid in the tip. This can result in liquid entering the barrel and/or piston. This liquid may carry over and contaminate subsequent samples. In addition, it is possible that certain solutions may damage the piston (see #2). If this happens, the pipettor should be disassembled, cleaned, and dried (please inform your instructor).

2. **DO NOT** use **volatile** or highly **corrosive** liquids. Use of highly volatile organic solvents will prevent the tip from filling completely, due to their high vapor pressure. Highly corrosive liquids such as concentrated acids may cause corrosion of the stainless steel piston. The pipettor should be disassembled, cleaned and dried after such use.

3. **FOR ACCURACY** when making dilutions, always pipet the largest volume into your tube or flask first. Then pipet the smaller volume into the larger volume. When adding a small volume to a larger volume, **never dispense the sample onto the side of the tube or flask**. Rather, always submerge the end of the pipette tip into the solution before dispensing the smaller volume.

Operation of Pipettors:

1. Set the desired volume by turning the volume adjustment knob until the correct volume shows on the digital indicator. For **best** precision, approach the desired volume by **dialing downward** (at least one-third revolution) from a larger volume setting.

To accurately set the desired volume on the pipettor, it is important to recognize the position of the decimal point. On the **small-volume model**, the decimal point is between the middle and lowest dials, with tenths of microliters indicated in color or by a line. On the **mid-volume model**, the decimal point is absent and two-tenths of a microliter subdivisions are indicated on the lowest dial. On the **large-volume model**, the decimal point is either between the top and middle dials, with milliliters indicated in color and hundreds and tenths of microliters indicated in black on the lower two dials, or missing so that the dial that shows 1000 µl with two-tenths subdivisions .

2. Attach a new disposable tip to the shaft. Press firmly with slight twisting to insure a positive, airtight seal. Loose tips can result in sample leakage or the tip falling off at an inappropriate time.

3. *Slowly* depress the plunger to the first positive stop (where you feel stronger but not total resistance). This is the calibrated volume displayed on the digital readout.

4. Hold the pipettor vertically and immerse the tip just below the surface (a few mm) of liquid.

5. Allow the push button to return **slowly** to the up position. **Never permit it to snap up.** This can result in liquid being pulled into the barrel and/or piston, causing the types of problems you have been cautioned about above.

6. Wait several seconds to insure that the full volume is drawn up into the tip. More viscous samples or very fine tips require a longer period of time.

7. Withdraw the tip from the sample liquid. If any liquid remains on the tip exterior, wipe it with a Kimwipe, taking care not to touch the tip opening, OR drain it by touching the container's side.

8. To dispense the sample, depress the plunger slowly to the second stop. Always assess that all the liquid has been dispensed before removing your pipettor (viscous solutions may take several seconds to be completely expelled from the pipette tip).

(2) DILUTIONS

Please read **Appendix I. Concentration and Dilution** before coming to lab. In this exercise, you will be making a series of dilutions of a 2 mg/ml bovine serum albumin (BSA) stock solution using Tris buffer. These dilutions will provide your protein concentration standards, which you will use to determine the concentration of a protein solution of unknown concentration, so accuracy is important. Serum albumin is an important transport protein in all mammals, acting as a carrier for several substances in the blood, most notably fatty acids. BSA, in particular, is frequently used as a source of protein for concentration standards, as it can be effectively isolated from a source that is both abundant and readily available (cow serum) and is therefore less expensive.

(3) UV/VIS SPECTROSCOPY

Please read **Appendix II. Spectroscopy** on spectroscopic principles. UV/Vis spectroscopy is a very valuable biochemistry laboratory tool for assaying, directly or indirectly, the concentration and concentration changes of various biomolecules. Using enzyme proteins as an example, the concentration changes of substrate or product molecules in the presence of the enzyme provide important data about the enzyme's activity. We will use UV/Vis spectroscopy to do this type and other types of measurements in several experiments this semester, so to understand what we will be doing later, it is important for you to become familiar with the principles involved now.

We will be using Cecil spectrophotometers in these and other experiments throughout the semester. Therefore, you will want to use today to learn how to use the instruments in preparation for other experiments during the semester. The software-spectrophotometer interface is Windows based and fairly intuitive. You will have opportunity to ask questions following a brief demo of the instrument, and the instructor and laboratory assistant will be able to help you if questions arise from your efforts to learn about the instrument.

BRADFORD ASSAY

The Bradford spectrophotometric assay (Bradford, M.M. 1976) provides for the indirect measurement of protein concentration in solution and is a commonly used method. In fact, you may have already used it, but, if not, you will use it today and in later experiments this semester.

As an *indirect* measurement of protein concentration, the Bradford spectrophotometric assay does not measure the light absorbed directly by the protein in solution. Rather, the assay measures the light absorbed by a specific dye that absorbs more of certain wavelengths of light (and less of others) when bound to proteins then when it is not. In simple terms, the physical interaction of the dye and protein in solution results in an observable change in color. This assay is an example of a *dye-binding assay* and, as such, its fidelity in measuring protein concentration is dependent upon the physical and chemical conditions influencing the interaction between protein and dye.

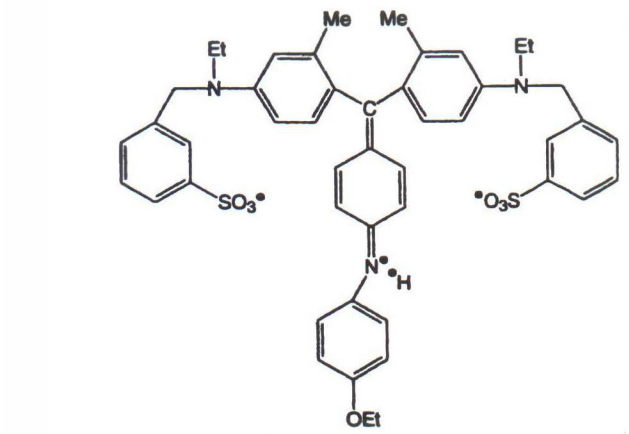


Figure 1. Structure of the blue ionic form of CBB.

Taken from Chial *et al.* 1995

In the Bradford assay, the dye used is Coomassie Brilliant Blue G-250, a sulfated compound (Figure 1). When not bound to proteins, the dye exists largely in the red form (A_{max} 465 nm). The blue form of the dye (A_{max} 595 nm) carries a (-) charge and interacts with (+) charges on proteins to form a complex (Chial *et al.* 1995). Increasing the amounts of the complex (which, when the dye is not limiting, depends upon the amount of protein) results in an increase in the absorbance at or near $A_{max.}$ This reaction is very rapid and reproducible. Samples are stable for up to one hour at room temperature.

The increase in absorbance resulting from interaction of the dye with protein is monitored using a spectrophotometer. To calibrate the absorbance to protein concentration, a series of protein standards are assayed and a plot of absorbance verses protein concentration (standard or calibration curve) is generated. This graph is used to determine the concentration of proteins in experimental samples based upon the absorbance of those samples.

EXPERIMENTAL PROCEDURE

Materials provided:

Tris buffer: [10 mM Tris-HCl (pH 7.0)]
Bovine serum albumin (BSA) stock solution: [2 mg/ml BSA in Tris buffer (pH 7)]
Bradford reagent; [0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid]
Mock Bradford solution; [4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid]

1. Obtain some stock solution of BSA. In microfuge tubes, prepare a series of BSA solutions of varying concentration by diluting the 2 mg/ml BSA stock solution with Tris buffer. You will need ~200 µl of each dilution at the following concentrations; 0.05 mg/ml, 0.1 mg/ml, 0.2 mg/ml, 0.3 mg/ml, 0.4 mg/ml, 0.5 mg/ml, and 0.6 mg/ml. **Be sure to label each tube** (compound, concentration, your initials, date). *Keep the unused portion of your samples at 4°C for Expt. 3.*

2. Add 2.5 ml of Bradford reagent to a separate test tube or cuvette for each of your samples and label them appropriately. Consider the value of determining the concentration of one or more dilutions of your unknown sample as well as the undiluted ("neat") unknown sample. Prepare your samples by adding 50 µl of each protein sample (diluted standard or unknown) separately to the Bradford reagent in the appropriately labeled tube. Mix the tubes by gentle inversion several times, and let the color develop for 5 min. Observe and record the color change of your standard samples as a function of protein concentration. By comparing your unknown to your protein standards, determine as closely as you can the concentration of your unknown by eye.

3. While the color is developing set up the spectrophotometer. Prepare a blank sample by mixing 50 µl Tris buffer with 2.5 ml Bradford reagent and another by mixing 50 µl Tris buffer with 2.5 ml Mock Bradford solution.

4. You will need to determine which portion of the UV/vis spectrum, specifically which wavelength, will be useful for following the dye bound by protein. Using your Mock Bradford blank sample in a plastic cuvette, set the background for the spectrophotometer. Take a full spectral scan of your Bradford reagent blank prepared in step 3. When your standard samples have fully developed, take a full spectral scan of the most concentrated standard you prepared.

5. Based on your results, chose a single wavelength suitable to analyze the results of your dye binding assay. Measure and record the absorbance of each standard and unknown sample at your chosen wavelengths using plastic cuvettes. These data may be obtained from spectral scans or from single wavelength readings. In either case, you must consider your data in light of the background absorbance of your "no protein" sample.

6. Prepare a plot of the values of absorbance above background verses protein concentration for the standard samples, and fit the data to a line by linear regression. Using the resulting equation for this line and the value of absorbance above background for unknown sample, determine the protein concentration of your unknown.

DATA ANALYSIS

For Expt. 1 you are to write a short lab report (see page 7). Using the instructions on pages 5-6, prepare a type-written report that includes only the Material and Methods and Results sections, plus answers to the questions below. Include *all* of the data obtained (in a table), a standard calibration plot figure with relevant equations, and the concentration of your unknown protein sample.

Questions:

1. How close was your determination of your unknown protein concentration by eye to the determination by Bradford assay? What did you learn from this exercise?

2. How appropriate is BSA as a concentration standard protein, considering standard calibration plots are often used to determine the concentration of proteins other than BSA? (see Chial *et al.*)

3. If you measured the concentration of a dilution of your unknown sample, what did you learn from this measurement?

References: Bradford, M.M. (1976) *Anal. Biochem*. **72**, 248-256**.**

Chial, H.J., Congdon, R.W., and Splittgerber, A.G. (1995) *J. Chem. Ed.* **72**, 76-79.

EXPERIMENT 2. Amino Acid Composition of a Dipeptide

Much can be learned about a protein or peptide by determining its amino acid composition (and sequence). To determine the amino acid composition, all peptide bonds in a purified protein must be cleaved (either enzymatically or with strong acid or base) and the individual amino acids must be separated, identified, and quantified. Thus, there are three aspects to this type of experiment; 1) **the reaction chemistry**, 2) **the separation** of the reaction components, and 3) **the assay** or analysis of the outcome (identification/quantification).

For **the reaction chemistry** of this experiment, we will be using enzymatic hydrolysis of peptide bonds. This approach is advantageous because acid hydrolysis tends to destroy some amino acids. There are many enzymes, collectively called *proteases* or *peptidases*, that cleave peptide bonds. Some are *endopeptidases*, in that they cleave internal peptide bonds; others are *exopeptidases*, in that they cleave N- or C-terminal peptide bonds. In this experiment, we will use the enzyme **carboxypeptidase A**, which cleaves C-terminal peptide bonds, to cleave the one peptide bond present in a purified dipeptide of unknown amino acid composition.

The reaction chemistry of our experiment depends on the activity of an enzyme preparation (presumable highly purified). Because enzymes are labile molecules and can lose their activity (i.e. when they denature), we will adopt an experimental strategy that implicitly reveals the status of our enzyme's activity along with the experimental result. We will utilize a *comparative* approach, by separating and assaying dipeptide "treated" and "untreated" with enzyme. We expect that the enzyme activity will result in a change to the treated dipeptide. By including the "untreated" reaction as a **control**, we would observe no change if the enzyme activity were lost or absent.

The **separation** of molecules of interest from uninteresting ones is a fundamental tool to biochemical research. In this experiment, separation of amino acids will be done using simple paper chromatography to illustrate general principles of separations. All chromatographic techniques are based on the principle that the solute of interest partitions in a characteristic way between a *stationary phase* and a *mobile phase*. The mobile phase (a gas or liquid) moves the sample solute through a region containing the stationary phase (a liquid or solid). The theory and practice of paper chromatography are very similar to those of thin-layer chromatography (TLC), which you used in Organic Chemistry lab. In paper chromatography, a piece of paper composed of cellulose is extensively hydrated, so solutes (amino acids here) partition between the immobilized water (stationary phase) and the solvent used to run the chromatograph (mobile phase). We will see additional application of the principles of this fundamental tool of biochemistry in the separation and purification of an enzyme protein in Expt. 3.

The **assay** of our experimental outcome involves the **identification** of the amino acids (we will not be quantifying the results of this more qualitative experiment). The first step in identification of the amino acids involves their detection. Detection of amino acids is accomplished by treating the paper with *ninhydrin*, which turns purple upon binding the free primary amino groups of amino acids. Consult your organic chemistry textbook to review the reaction chemistry of ninhydrin. Assigning an identity to each unknown amino acid is accomplished by including on the chromatograph samples containing a known amino acid and comparing the relative mobilities of solutes in your unknown sample with those of known standards. The relative mobility of a molecule is indicated by the R_f value.

$$R_f = \text{Distance (origin to sample spot)} \div \text{Distance (origin to solvent front)}$$

R_f values vary with solvent, type of stationary support (e.g., cellulose, silica gel) and temperature.

EXPERIMENTAL PROCEDURE

Materials provided:

Enzyme buffer: [25 mM Tris-HCl (pH 7.5), 500 mM NaCl]
Unknown dipeptide; [2 mg/ml in enzyme buffer]
Carboxypeptidase A (500-1,000 units/ml)
1.0 M acetic acid
Amino acid standards [1 mg/ml in enzyme buffer]
Whatman 3MM chromatographic paper, 20x20 cm
Chromatographic solvent [60:40 acetonitrile:0.1 M ammonium acetate] and jar or chamber
Ninhydrin solution [0.2% ninhydrin in ethanol]

1. Set up three tubes. First, in each of two microcentrifuge tubes, place 100 µl of unknown dipeptide solution. To one of these tubes, add 5 units carboxypeptidase A and, to the other tube, add a volume of enzyme buffer equal to the volume of enzyme added to the first tube. As a control for amino acids contributed from the enzyme source, place 100 µl of enzyme buffer in a tube and add 5 units of carboxypeptidase A. Mix all tubes gently by tapping them with your finger. Incubate for one hour at 37°C in a water bath.

2. For the following step, **wearing laboratory gloves**. Amino acids deposited on your chromatography paper from the skin of your fingers are abundant enough to be detected by ninhydrin as fingerprints (this is used in forensic science!). **Prepare** a piece of Whatman 3MM paper for chromatography. Lightly mark the origin 2 cm from one edge (the bottom) of the paper with a **pencil**. Indicate with dots where each sample and standard will be loaded onto the paper at the origin, keeping the dots at least 1.5 cm apart. You will need 4 lanes for your sample and one lane each for each of the amino acid standards. Label each dot. Also **prepare** the solvent chamber by adding enough solvent to reach 1 cm height in a large glass jar or plate chamber. Close the lid so that the solvent vapor and liquid phases equilibrate.

3. Add 50 µl 1.0 M acetic acid to all three tubes, including the reaction tube and the control tubes. **Mix.** Acetic acid stops the enzyme-catalyzed reaction and causes the enzyme to precipitate.

4. Spin each tube in the microcentrifuge for 10 min at **3000 rpm** to pellet any precipitate.

5. Carefully remove 50 µl of the supernatant from each tube, taking care not to disturb any pellet at the bottom of the tube.

6. Load 2 µl of each amino acid standard. Based on the amount of standard used, determine about how much unknown sample you will need. Load two different amounts of each unknown sample (± enzyme) to the paper separately at two dots. Let loaded samples air-dry briefly.

7. Roll the paper into a cylinder and staple the edges, taking care not to let the edges touch if using a large jar. This is easier if one person holds the paper in a cylinder, and another staples. Secure paper to insure it remains upright if you are using a plate chamber. **Make sure the solvent in the chromatography chamber is below the origin on the paper**, then drop the paper into the solvent chamber. Let the chromatograph run for approximately 1 hour, or until the solvent front is 1-2 cm from the top of the paper.

8. Remove the paper from the chamber, quickly but carefully remove the staples and mark the solvent front lightly with a pencil. Let the solvent air-dry. When dry, spray evenly with ninhydrin solution in the hood. The paper should be damp, but not dripping, with ninhydrin solution. Place the paper upright in the oven at 110°C until the purple spots develop (~ 15 minutes).

9. Calculate R_f values for the standards and unknown sample. If the spots are broad, measure from the middle of the spot.

DATA ANALYSIS

For Expt. 2 you are to write a short lab report (see page 7). Using the instructions on pages 5-6, prepare a type-written report that includes only the Material and Methods and Results sections, plus answers to the questions below. Include *all* of the data obtained (R_f values in a table) and the amino acid composition of your unknown dipeptide sample.

1. Look up the substrate specificity of carboxypeptidase A. You might find this in one or more of the books on enzymes in the library, another biochemistry text, or on web resources. Could you use this enzyme to cleave the C-terminal peptide bonds of any peptide? Explain your reasoning.

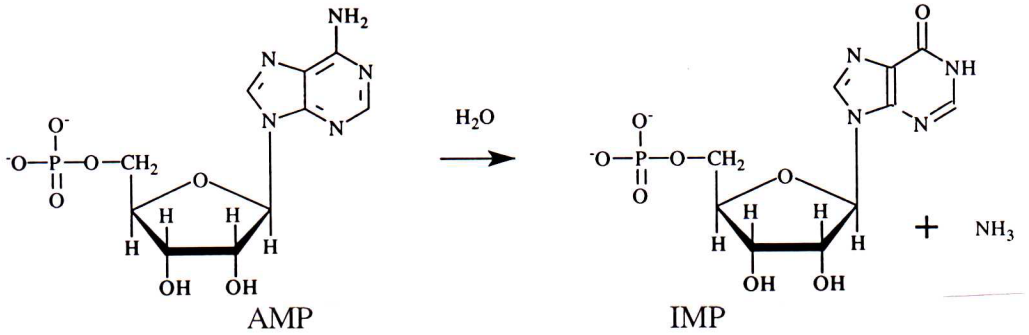
2. Can you determine or predict the amino acid sequence of your dipeptide? How could you change this experiment to determine the sequence of these two amino acids in the dipeptide?

EXPERIMENT 3. Purification of AMP Aminohydrolase

In Expt. 2, you learned that the separation of molecules from each other is a fundamental tool used in biochemical research. Separations can be employed for analytical purposes (as in Expt. 2), which often involve small amounts of material, or for **preparative** purposes, which involve larger amounts of material and aim to recover relatively pure molecules for additional study. In biochemistry, a common goal for employing separation techniques is the purification of a protein of interest for preparative purposes. This is a daunting task! The development of a successful procedure for purifying the protein of interest from the hundreds of other proteins in the starting material often requires trying a series of some of the variety of separation methods in different orders and/or combinations. Each separation method generally exploits a specific physical property of the protein of interest that, ideally, is not shared with too many other proteins in the starting material. In an effective purification, the relative purity of the desired protein is increased by combining two or more separation methods, each of which generally exploits a different physical property of the protein. With each additional step, however, the final amount of protein recovered generally becomes less.

Effective protein purification usually requires at least one column chromatography separation. The general principles of chromatographic techniques were introduced in Expt. 2. Remember, all chromatography is based on the principle that the solute of interest partitions in a characteristic way between a *stationary phase* and a *mobile phase*. The mobile phase (a liquid in this experiment) moves the sample solute (protein of interest) through a region containing the stationary phase (a solid resin in this experiment). The type of chromatography used in this experiment is called affinity chromatography, as it exploits the high binding specificity and affinity the desired protein has for the stationary phase. The binding of the protein of interest to the stationary phase has the potential to very effectively remove it from the hundreds of other proteins in the mobile phase. If no other proteins in the starting material share the affinity the desired protein has for the stationary phase, affinity chromatography can, very powerfully, permit complete purification.

The goal of this experiment is to purify the enzyme AMP aminohydrolase from chicken muscle. AMP aminohydrolase (or adenylate deaminase) is an abundant enzyme in contracting muscle, and is involved in the degradation of purine nucleotides. It catalyzes the hydrolysis of adenosine 5'-monophosphate (adenylate, AMP) to inosine 5'-monophosphate (IMP) plus ammonia.



The column chromatography step that you will use has been selected based on the specific binding properties of AMP aminohydrolase. Often, a researcher can predict that a specific enzyme might bind to a chromatographic resin (stationary phase) if the ligand attached to the resin resembles the enzyme substrate. The enzyme binds to a substrate or substrate analog ligand immobilized on the insoluble matrix of resin. It can then be *washed* free of other proteins and *eluted* by a change in the mobile phase that will alter the affinity of the protein for the resin-ligand and cause its release.

The resin used in this experiment is phosphocellulose, which contains a high number of negatively-charged phosphate groups attached to cellulose. It is most frequently used as a resin for ion-exchange chromatography. However, since AMP aminohydrolase binds to the substrate AMP, which contains a negatively-charged phosphoryl group, one might predict that it will also bind to this resin. If it binds to this substrate analog, one might predict that the enzyme could be eluted by pyrophosphate (PP_i) (an inhibitor of the enzyme), which decreases the affinity of the enzyme for its substrate (AMP). Pyrophosphate also partially mimics the phosphate groups attached to the cellulose resin. In this experiment, you will characterize the effectiveness of phosphocellulose as a matrix for affinity purification of chicken muscle AMP aminohydrolase.

EXPERIMENTAL PROCEDURE

As biochemical purification is often fairly extensive, Experiment 3 will be done over the course of the next three weeks. In the **first** week, you will prepare the protein extract from chicken muscle and chromatography resin, and run a brief exercise intended to familiarize you with the Pharmacia chromatography system we will be using in the second week. In the **second** week, you will assemble and run the chromatography system to purify (hopefully) AMP aminohydrolase from your chicken muscle extract. This week concludes with assaying the fractions you collect from the column for enzyme activity, pooling those of highest activity, and dialyzing them to remove the pyrophosphate eluant from your purified enzyme sample. In the **third** week, you will assay the major fractions of the purification for protein concentration and enzyme activity to help assess the method. You will also analyze these same fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to assess relative complexity of the proteins in each sample, and the relative purity and molecular weight of AMP aminohydrolase (see Appendix VI).

Week 1

This week, your working group will be divided to provide a representative to a task groups that will accomplish the three tasks of this week; preparation of chicken protein extract, preparation of chromatography resin, and preparation for using the chromatography system next week. As your working groups representative to one of this week's task groups, it will be your responsibility to become involved in the task, learn all that is necessary to do it independently, and report back to your group what you learned. Your working group partners will share a similar responsibility to explain to you how to do what they learned to do.

Materials provided:

- Chicken breast muscle (from the local grocery store)
- Extraction buffer: [0.18 M KCl, 54 mM K_2HPO_4, 32 mM KH_2PO_4 (pH 6.5)]
- Waring blender
- 250 ml centrifuge bottles
- Funnel
- Glass wool
- Oakridge 40 ml screwcap polypropylene centrifuge tubes
- Phosphocellulose resin; pre-swelled in dH_2O overnight
- Basic resin wash solution:[0.05 M NaOH, 0.5 M NaCl]
- Acidic resin wash solution: [0.1 M HCl]
- Glass columns
- Buffer A: [25 mM TrisHCl (pH 8.1)]
- Buffer B: [25 mM TrisHCl (pH 8.1), 500 mM NaCl]
- protein standards: [0.15 mg/ml equine myoglobin, 0.38 mg/ml conalbumin, 0.46 mg/ml chicken albumin, 0.38 mg/ml soybean trypsin inhibitor, 25 mM TrisHCl (pH 8.1)]

CAUTION: To prevent proteolysis and preserve enzyme activity, keep all enzyme fractions and all solutions at 4˚C (in the refrigerator or on ice) as much as possible during extraction and purification. Carefully label all your samples.

PREPARATION OF CHICKEN BREAST EXTRACT - Extract preparation will be done in batch and will be the responsibility of a group of four that includes one member of each working group.

1. Quickly cut up one partially thawed chicken breast into small pieces (1 cm). Place 50 g in a Waring blender and homogenize with 200 ml of **cold** extraction buffer at high speed with five 15-sec bursts. Make sure the blender lid is on tight! Immediately pour homogenate into prechilled 250 ml centrifuge bottles and keep on ice.

2. **Important: balance centrifuge bottles to equal weights** using Harvard Trip balance. Centrifuge the homogenate at 6,000 x g (check specifications for rotor used) for 15 min at 4°C.

3. Pour the supernatant through a funnel with a "filter" of glass wool into a beaker on ice. Discard the pellet. Record the volume of filtrate and aliquot an equal volume into four polypropylene Oakridge tubes. Seal screw cap and store tubes in the -20°C freezer until next week.

PREPARATION OF PHOSPHOCELLULOSE RESIN AND COLUMN (adapted from directions of Sigma Chemical Company) - The resin preparation will done as a batch, and will be the responsibility of a group of four that includes one member of each working group.

1. Obtain the pre-swelled phosphocellulose resin (3 g = ~20 ml wet resin) and decant water.

2. Add a volume of water 5 times that of the wet resin, stir and allow to settle for 30-45 minutes. When most of the resin is settled, decant. Record the resin volume.

3. Suspend resin using 5 times the resin volume of basic wash for 10 min. Pour slurry into a course scintered glass funnel under gentle suction (~1 resin volume/5 min). Continue until all of the slurry has been added and follow with a wash of 5 times the resin volume of deionized water at 1-5 resin volumes/min.

4. Use acidic wash to resuspend resin and remove it from the funnel. Soak in a total of 5 times the resin volume of acidic wash for 10 min. Pour slurry back into the course scintered glass funnel and wash with 10 times the resin volume of water until flow-through shows a pH of ~5.

5. Wash with 5-10 times the resin volume of extraction buffer until the pH of the flow-through reaches that of extraction buffer (~ 6.5). Resuspend in extraction buffer and transfer to a beaker.

6. Allow the resin to settle and decant all but one resin volume of buffer.

7. (To be done by working group) Mix resin and buffer into a slurry (it will be 1:1 resin:buffer slurry, see step 6), and measure a volume of slurry equal to twice the amount of resin bed ($\geq$ 2 ml) desired in your column. Dispense into your column, cap and store at 4°C. Record bed volume.

PREPARATION FOR USING THE PHARMACIA PUMP AND COLLECTOR SYSTEM - Becoming familiar with how to use the peristaltic pump and fraction collector in preparation for today's chromatography exercise will be the responsibility of a group of four that includes one member of each working group. The exercise itself is to be done by each working group.

1. Identify how to assemble the tubing, column, and fraction collector and become familiar with its plumbing and operation. Determine how to make a two solution gradient (see instructor demonstration).

2. Once assembled as a working group, instruct your group on what you have learned about the equipment you will be using for column chromatography.

3. (As a working group) Fill the system line with buffer and eliminate air bubbles. Set flow rate to be constant at about 1.5 ml/min.

4. Attach the ion exchange column to the system line and wash the column, removing any air from tubing with buffer A.

5. Before starting, set your fraction collector to begin collect 3 ml samples.

6. Place inlet tubing into your protein standards solution without introducing any air (a slight squeezing of the tubing can help achieve this). Run the pump until about 2 ml of your sample has entered the tubing.

7. Pause the pump and transfer the inlet tubing to buffer A. Avoid introducing any air. Run the pump for about two minutes.

8. Pause the pump and transfer the inlet tubing to the gradient buffer set up without introducing any air. Run the pump for 10 minutes.

9. Pause the pump and transfer the inlet tubing to buffer B. Avoid bubbles. Run the pump for 6 minutes.

10. Pause the pump and transfer the inlet tubing to buffer A. Avoid bubbles. Run the pump for 6 minutes.

11. Determine the amount of protein in each of your fractions by either direct absorbance or through use of the Bradford assay (see Expt. 1).

12. Plot the protein concentration or absorbance as a function of fraction number. Assess the separation of the various proteins in the standard mixture.

Week 2

This week you will work with your working group, and you will have a lot to do, so preparation will be especially helpful. You will prepare your chromatography system for your purification method and prepare the system by filling the line with the correct initial buffer. You will assemble the column-flow adaptor assembly and attach it in line with the chromatography system. You will prepare and apply your sample to the chromatography system, running a sequence of buffers to isolate and purify (hopefully) AMP aminohydrolase from chicken muscle extract. Finally, you will assay and determine which fractions have the highest enzyme activity, pool and dialyze them (overnight) to remove the pyrophosphate eluant from your purified enzyme sample.

Materials provided:

- Extraction buffer: [54 mM K_2HPO_4, 32 mM KH_2PO_4, 0.18 M KCl (pH 6.5)]
- Column wash buffer: [0.45 M KCl, 25 mM MES (pH 6.5), 1 mM 2-mercaptoethanol, pH adjusted with solid Tris]
- Pyrophosphate elution buffer: [25 mM potassium pyrophosphate in column wash buffer]
- Flow adaptor
- Fraction collection test tubes
- Substrate buffer: [0.15 M KCl, 50 mM MES (pH 6.5), pH adjusted with solid Tris]
- Assay solution: [5 mM AMP in assay buffer]
- Spectrophotometer
- Dialysis tubing or cassettes
- Dialysis buckets, stir bars, and stir plates

SAMPLE PREPARATION - As this takes a while and should not be rushed, you will want to start this at the beginning of the lab.

1. Completely thaw chicken protein extract of week 1 on ice.

2. Check balance of centrifuge tubes to insure or adjust to equal weights using Harvard Trip balance. Centrifuge the homogenate at 6,000 x g (check rotor specifications) for 15 min at 4°C.

3. Remove supernatant to new tube, on ice! Measure the extract volume, and retain 0.5 ml aliquot in a microcentrifuge tube for analysis of activity.

COLUMN PREPARATION - While your extract is thawing, prepare your column.

1. Fill the column with excess cold extraction buffer and allow several tens of milliliters to flow through to settle the resin bed further (gravity only goes so far).

2. Make sure there is ample extraction buffer in the column tube (5 times resin bed), and allow any resin to settle that might have been disturbed during column filling.

3. Fit the flow adaptor to the column taking care to avoid and eliminate bubbles. (See demo.)

SYSTEM PREPARATION - While the extract is being prepared, assemble the Pharmacia pump and collector system for purification of AMP aminohydrolase.

1. Completely fill tubing with buffer to be used, i.e. extraction buffer, and purge system of air.

2. Make sure *column-flow adaptor* lines are filled with extraction buffer and free of air.

3. Install the column-flow adaptor assembly in the system; avoid bubbles upstream of the column.

4. Run extraction buffer slowly through the system for several minutes to purge any air downstream of column and to test for any problems.

5. Prepare a separate beaker with column wash buffer.

6. Prepare apparatus to create a two-buffer gradient of column wash buffer and pyrophosphate elution buffer.

RUNNING THE SYSTEM - As a group, review from week 1 the operation of the chromatography system and the volume of your column resin bed (arbitrarily defined as 1 column volume or CV). Determine a desirable flow rate. Using the guidelines below, discuss your separation strategy and prepare a plan for your separation. Remember, you have a single inlet line, so think about the different solutions you need to have on hand to achieve the outcome you desire (i.e. you will need to move the inlet line between different buffers). Check your plan with the instructor or a teaching assistant before beginning.

1. Prepare your plan to use the pump for five steps, using your selected flow rate:
   a - Load extract (in extraction buffer) into system, *measure/record the extract volume.*
   b - Extraction buffer wash; should be 5 CVs minimum.
   c - Column wash buffer wash, should be 5 CVs minimum. The A_{280} of your post-column flow should be 0.01 or lower if you have washed your column sufficiently well. If not, you may wish to extend your wash step before eluting the column.
   d - Gradient (0-15 mM PPi) elution step using column wash buffer and elution buffer (column wash buffer containing 25 mM potassium pyrophosphate, 2-4 CVs).
   e - Final wash with column wash buffer to complete protocol, 5 CVs.

2. Set inlet line into protein extract (on ice) and start (run) the pump and collector.

Note on loading extract volume: Because the pump system is not precisely calibrated, you must measure initial and final volumes to determine the exact volume delivered to the column resin. Watch during your extract loading to insure that you do not run out of extract or have too much left unloaded before the second step. **IF** you have protein extract that remains unloaded, measure the volume remaining. It will be important to subtract this amount from the initial volume you intended to load, or you will overestimate the enzyme activity exposed to the column.

If it appears you will **run out** of extract, **PAUSE** the pump. Switch the inlet line to the next buffer and resume the separation. If it appears you will have too much **unloaded** extract, continue running the pump to load what you want but be sure to make a record of the amount loaded. Once it is adequately loaded, **PAUSE** the pump to switch the inlet line to the next buffer.

3. Pause the pump and switch the inlet line to the other buffers or gradients at the appropriate times as per your plan. Run steps for the times planned given the pump flow rate you determined.

Note on pausing to switch buffers: Stop the pump to move the inlet line to a new buffer vessel, which will prevent the introduction of air to your column. If necessary, squeeze the flexible pump tubing to extrude air before placing the inlet tube opening into a new buffer.

3. Operate the fraction collection using a strategy that will separate your enzyme from others.

a - Extract flow-through: Using large fractions is convenient for collecting all of your extract flow-through (post-resin extract), but you will need to use smaller fractions to obtain a small but enriched sample of purified enzyme. Thus, you may wish to change fraction size during your collection. If your extract flow-through exists as more than one fraction, pool these to create your "flow-through" fraction and for analysis. As the extract will take some time to get through the system and to the fraction collector, you can delay the start of your collection appropriately. In addition, it will take some time for the extract in the system line to pass entirely through the system once you begin the first wash step, so you should not stop collecting when the first wash begins. Overall, it would be better to get all your flow-through extract and have some extra extraction buffer dilute your flow-through than to recover only part of your flow-through.

b - Wash flow-through: Collect the protein eluted with high salt column wash buffer (optional). This will allow you to assess if any of the enzyme activity is washed off with this buffer change. You can also check absorbance to see if you have washed enough.

c - Elution of column: Collect several and smaller fractions during the elution to capture and fractionate all of your sample. The less volume you use for the elution, the smaller the volume you want these fractions to be. Remember there is a time lag between application of your gradient and when it reaches the resin, but here it doesn't hurt to collect a few extra fractions before and after the program time of the elution to insure that you recover all of the interesting fractions.

Note on chilling fractions collected: As soon as physically possible, **label** and place on ice each fraction tube collected from the program method. This preserves enzyme activity. One way to doing this is to set an ice bath in the collector tube holder itself. Alternatively, you may place tubes into an ice bucket.

4. Record the results from your separation attempt (not just the method plan).

SYSTEM AND COLUMN CLEAN UP -

1. When the program is finished, remove the column from the system line, and clean the system by purging distilled water through the lines extensively for several minutes. Excessive washing is critical to avoid clogging the fraction collector dropper. When the system is adequately washed, disassemble the pump head, leaving the inlet lines in distilled water, and turn the system off.

2. The column-flow adaptor assembly can be disassembled and the resin mixed into a slurry and decanted to a "waste" beaker. Both the column and flow adaptor should be rinsed extensively with distilled water and placed aside to dry.

POST-SEPARATION ACTIVITIES - You will need to do several things once completed.

1. Pool extract "flow-through" fractions, mix, and measure the total flow-through volume. Aliquot a sample to a microcentrifuge tube on ice.

2. Measure and record the volumes of the extract loaded on the column ($V_{initial}$ - $V_{remaining}$) and the flow-through obtained afterward, if you have not already.

3. Perform the enzyme assay on the aliquot of protein extract and pooled flow-through fractions, as well as each of the elution fractions (and the column wash buffer fraction(s) if this was done) and the mixture of pooled elution fractions, as directed below.

4. Freeze the remainder of the extract and flow-through aliquots for determination of protein concentration and electrophoresis next week. See below for treatment of the elution fractions.

ENZYME ASSAY OF COLUMN FRACTIONS - Please read **APPENDIX II: Spectroscopy** and review the use of the Beer's-Lambert law before doing the following. AMP aminohydrolase activity is determined by recording the change in absorption at 265 or 290 nm due to the difference between the spectra of AMP and IMP, both of which absorb UV light. There is much more absorption at 265 nm than at 290 nm. In fact, at high concentrations of AMP, the level of A_{265} is too high for the spectrophotometer to detect a change upon addition of enzyme. Therefore, at high [AMP] (greater than 0.2 mM), measure the initial **increase** in A_{290} per minute and divide by a factor of 0.12 (the difference in extinction coefficients of AMP and IMP). At low [AMP] (lower than 0.2 mM), measure the initial **decrease** in A_{265} per minute and divide by a factor of 8.76.

1. Transfer exactly 1 ml of assay solution (5 mM AMP is saturating substrate) to a quartz cuvette and zero the absorbance at 290 nm (**make sure** the solution is at room temperature).

2. Add an aliquot of enzyme fraction (10-25 µl), rapidly mix, and record the increase in A_{290} for several minutes (or until it becomes non-linear). *Make sure you use the same volume of enzyme from each elution fraction, so that you can compare the amount of activity in each fraction.*

3.Determine the initial linear rate (ΔA/min) and convert to enzyme activity [**units/ml sample = ΔA/min/(0.12 x ml sample)**] where one unit = 1 µmol/min substrate hydrolyzed in 1 ml assay mixture. Use the volume of enzyme fraction added in the denominator to determine its activity.

DIALYSIS OF PURIFIED ENZYME SAMPLE - Please read the section on dialysis in **Appendix IV. Enzymology** before starting this portion of the experiment.

1. Pool the major enzyme-containing fractions from the phosphocellulose column and mix gently. Measure and record the volume.

2. Assay the enzyme activity using an aliquot of the pooled fractions and record the result.

3. Remove an aliquot of the pooled fractions to a microcentrifuge tube, label and freeze. This sample will be included in the Bradford assays you will be doing next week, and serves to help you assess if there is any protein loss during dialysis.

4. **Wearing gloves**, place a dialysis cassette into column wash buffer to soak 1 minute. Introduce the pooled fractions into the cassette using a syringe; alternate introducing some sample with removing some air. After all sample has been introduced, remove all air from the cassette.

5. Insert cassette into a floatation aid and dialyze overnight at 4°C (in the cold room) against the wash buffer to remove pyrophosphate. This should be done with gentle mixing using a stir bar and a stir plate.

6. (THE NEXT DAY) Remove the dialyzed sample (dialysate) from the cassette using a syringe; alternate removing some sample with introducing some air. To avoid bacterial contamination however, **make your sample 0.02% sodium azide**. Store at 4°C; the pure, dialyzed enzyme should be stable for several weeks at 4°C.

Week 3

This week you will work with your working group. You will assay the major fractions of the purification for protein concentration and enzyme activity to help you assess the method. You will also analyze these same fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to assess relative complexity of the proteins in each sample, and the relative purity and molecular weight of AMP aminohydrolase.

Materials provided:

Substrate buffer: [0.15 M KCl, 50 mM MES (pH 6.5), pH adjusted with solid Tris]
Assay solution: [5 mM AMP, 0.15 M KCl, 50 mM MES (pH 6.5), pH adjusted with solid Tris]
Spectrophotometers
Tris buffer: [10 mM Tris-HCl (pH 7.0)]
Bovine serum albumin (BSA) stock solution: [2 mg/ml BSA in Tris buffer (pH 7)]
Bradford reagent; [0.01% (w/v) Coomassie Brilliant Blue G-250, 4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid]
Pre-poured, SDS-polyacrylamide gel (7.5 % acrylamide)
10X reservoir buffer: [1% SDS, 0.5 M Trizma Base, 1.9 M glycine] Dilute 1:10 for use.
5X Gel Loading Buffer (blue juice): [100 mM Trizma Base, 3.25% SDS, 50% glycerol, 1.7 mg/ml bromophenol blue] Add ß-mercaptoethanol just before use to 2.5% (v/v) final concentration. **Use the hood**; it stinks! Dilute 5-fold for use.
Gel Stain: [0.4 mg/ml Coomassie Blue R250, 40% (v/v) methanol, 5% (v/v) acetic acid]
Gel Destain: [10% methanol, 15% acetic acid]
Microcentrifuge
Protein gel electrophoresis apparatus and power supply

DATA COLLECTION FOR PURIFICATION TABLE

1. Assay as before your pooled dialyzed enzyme fraction, and all purification fractions not yet assayed (crude extract, flow-through, and pre-dialysis elution pool), for enzyme activity .

2. Assay all purification fractions for protein concentration using the Bradford assay (2.5 ml Bradford reagent + 50 µl protein sample - Expt. 1). Remember to generate a standard curve.

SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS - Please read **Appendix VI. SDS-PAGE** before starting this part. Each lab group should obtain and use or plan to share one pre-poured minigel (7.5% resolving gel with no upper stacking gel) to analyze their proteins.

1. Prepare molecular weight standards and your four protein samples (**1-**crude extract, **2-**flow-through from column, **3-**pooled, dialyzed enzyme fraction, **4** - protein mass standards). Determine the volume of protein sample necessary to load 5 µg of each onto the gel, as well as that for ~3 µg of SDS-PAGE molecular weight standards. For concentrated samples, it may be necessary for you to make a dilution in order to accurately pipet the desired amount. For each sample, aliquot the determined volume into its own microfuge tube. Add to each sample a volume of 5X Gel Loading Buffer equal to one fourth the volume of the sample aliquot. *Make sure fresh ß-mercaptoethanol has been added to the 5X buffer.* Heat samples for 5 min in a boiling water bath. *Note heating samples with ß-mercaptoethanol denatures and reduces all proteins.*

NOTE: The final volume of each sample need not be the same, but it is best if it is 10-30 µl. The gel wells hold ≥30 µl, and small samples (<10 µl) may evaporate upon heating. Adjust as needed.

CAUTION: ***UNPOLYMERIZED ACRYLAMIDE IS A NEUROTOXIN. ALWAYS WEAR PROTECTIVE GLOVES WHEN WORKING WITH ACRYLAMIDE GELS!***

2. Put on a pair of disposable gloves. Place gel plate into the electrode assembly and assemble. Use a buffer dam if only one gel is being run. Pour 1X reservoir buffer into upper buffer chamber formed by these two plate holders. *Make sure no buffer leaks out! If it does, reassemble the apparatus.* If the upper buffer chamber does not leak, fill the lower chamber up to the electrode at the bottom of the gel(s). Using the pipettor, apply each sample and protein standards into a separate well of the stacking gel. Record in your notebook which sample is loaded into each lane. *If possible, avoid loading sample in the outer lanes of the gel.* Avoid trapping large bubbles along the bottom edge of the gel plates.

3. Run the gel at 15 mA (constant current) per gel (30 mA per two gels). After the tracking dye has reached the bottom of the gel, turn the power off. Remove the plates and gently pry the plates apart. Place gel in stain for 1 hr (or overnight). Wash all parts of the gel apparatus thoroughly with soap and water and rinse with dH_2O.

4. (THE NEXT DAY) Transfer gel to destain solution until bands appear and the background is relatively clear. Rinse gel briefly in dH_2O (optional 3% glycerol prevents cracking).

5. Dry the gel between two sheets of cellophane (as demonstrated by the instructor).

6. Measure the distance migrated for the protein molecular weight standards and all bands of interest. See Appendix VI for determination of apparent molecular weight of your purified enzyme.

DATA ANALYSIS

For Expt. 3 you are to write, individually, a formal lab report (page 8). This will be handed in first as a draft, and critiqued by me and reviewed by two of your peers. Using the instructions on pages 5-6, prepare a formal type-written report. Include *all* of the data obtained; specifically a figure of your chromatographic separation, a table of the values of enzyme activity in your elution fractions, a table of raw data and corresponding values of enzyme activity and protein concentrations of your purification fractions, a figure or table or equation for your Bradford standards, a purification table (see below), a figure of your gel, and a figure of the plot for your protein molecular weight standards. Include in the purification table (see **Appendix IV. Enzymology**) the total volume, total amount of protein, total units of enzyme activity, specific activity, purification factor, and percent yield of enzyme in each of the purification fractions (**1-**crude extract, **2-**flow-through from column, **3-**pooled enzyme fraction before dialysis, and **4-**pooled, dialyzed enzyme fraction — indicate which fractions were pooled in fraction **4)**. All tables and figures should have legends and each component of all figures should be clearly labeled.

You may report your findings from this experiment as if you are the first group to report the purification of AMP aminohydrolase from chicken muscle OR as if you have discovered an improved, one-step purification for AMP aminohydrolase. Also, include answers to the questions below in the discussion in your report.

Questions:

1. How pure is the enzyme after phosphocellulose chromatography? Is this an effective chromatographic step to purify this enzyme? Identify your criteria.

2. What is the molecular weight of a monomeric subunit of this enzyme according to SDS-PAGE? What is its native molecular weight? How many subunits must it have? (see APPENDIX VI.)

3. How stable is the enzyme? Identify your criteria.

4. How does its purity compare with commercially available enzymes? (Hint: consult the Sigma or CalBiochem catalogs.) Could this be a potentially lucrative commercial method?

5. What do you think accounts for the enzyme activity lost during purification?

6. What else did you learn about this enzyme? Be specific.

Reference: Mahn, M.M. and Husic, D.H. (1989) *Biochemical Ed.* **17**, 94-96.

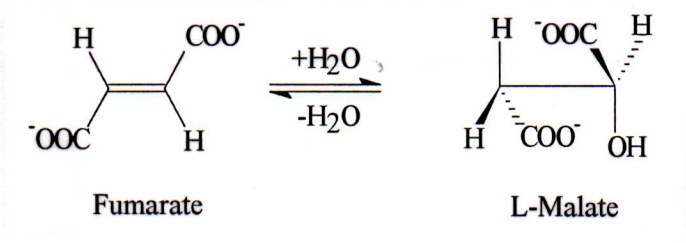
Boosman, A. and Chilson, O.P. (1976) *J. Biol. Chem.* **251**, 1847-1852.

EXPERIMENT 4. pH Dependence of Fumarase

Enzymes catalyze chemical reactions by changing the rate of a reaction but not its equilibrium. The catalytic effect of an enzyme is sensitive to the environment in which the enzyme functions. The native environment for many enzymes is the inside of cells, where conditions are established, maintained, and modulated by cellular mechanisms to control enzymatic activity. However, once a biochemist has purified an enzyme that is interesting to study (as you have just done in Expt. 3), she or he will most often modulate environmental variables likely to affect the activity of the enzyme *in vitro.* This is done for two reasons. First, sometimes the influence of a certain environmental variable on enzyme activity is interesting in and of itself. Secondly, routine parameters are modified to identify conditions for optimal enzymatic activity in the non-native environment of a test tube. This is done so that a large window of enzymatic activity is available to reveal the profound AND subtle effects of other interesting influences upon enzyme activity.

Since most biochemical reactions occur in the aqueous medium of the cell, and since many reactions involve proton transfers, pH is a very important variable in an enzyme-catalyzed reaction. In fact, biochemist often spend a great deal of time and effort thinking about and testing suitable buffers to control the effect of pH as a variable in the enzyme's environment. We have both of the previously mentioned reasons for understanding pH as an environmental variable. We are interested in the effect pH has on enzymatic activity, but we are also interested in learning the pH at which the enzyme has optimal activity, so that we can use that pH in experiments 5 and 6.

In this lab, you will explore the effect of pH on the catalytic activity of the enzyme **fumarase (fumarate hydratase)**. Fumarase catalyzes the hydration of fumarate to yield L-malate, one of the steps in the citric acid cycle.



The citric acid cycle is part of the process of cellular respiration, which serves to enzymatically oxidize the two-carbon fragments resulting from the oxidative breakdown of glucose and fatty acids to CO_2. The enzyme catalyzed conversion of fumarate to malate is reversible; both reactions are mediated by fumarase (we will see this in Expt. 5). The reaction can be inhibited (we will test this in Expt. 5). The reaction is also stereospecific (we will see this in Expt. 6) — the enzyme does not recognize the *cis*-isomer of fumarate (maleate) or the enantiomer of L-malate (D-malate).

The reaction catalyzed by fumarase can be followed spectrophotometrically because fumarate, with a carbon-carbon double bond, absorbs strongly in the near-ultraviolet (UV) range; malate does not absorb in this range. As fumarate is converted to malate (in the forward direction of this reaction), the absorbance by fumarate in the near-UV range decreases over time. Using this assay, the rate of this reaction is defined in the forward direction as the amount of fumarate used up per unit time. The rate in the forward direction can also be defined as the amount of malate formed per unit time. To explore the effect of pH on the activity of fumarase, you will use a spectrophotometer to measure changes in near-UV absorbance over time to determine the rate of fumarase activity in buffers at various pH values. From these data you will learn something about pH as an environmental variable affecting enzyme activity, and the optimal pH for fumarase activity.

To better understand the effect of pH on the activity of fumarase, you will need to know about the mechanism of fumarase action. Fortunately, the mechanism of this reaction has already been well characterized (see Weaver and Banaszak, 1996). It is an example of *general acid-base catalysis* (Fig. 1). In acid-base catalysis, acidic and basic chemical groups from the termini or amino acid side chains of the enzyme donate or accept protons from reactants to make the reactants better electrophiles or nucleophiles, respectively. The fumarase-catalyzed reaction involves a carbanion substrate intermediate, and acidic and basic groups are required for the generation of this intermediate. The reaction is therefore dependent upon the pH of the surrounding medium, because pH affects the ionization state (i.e. the protonation/deprotonation state) of acidic and basic groups (remember the Henderson-Hasselbalch equation).

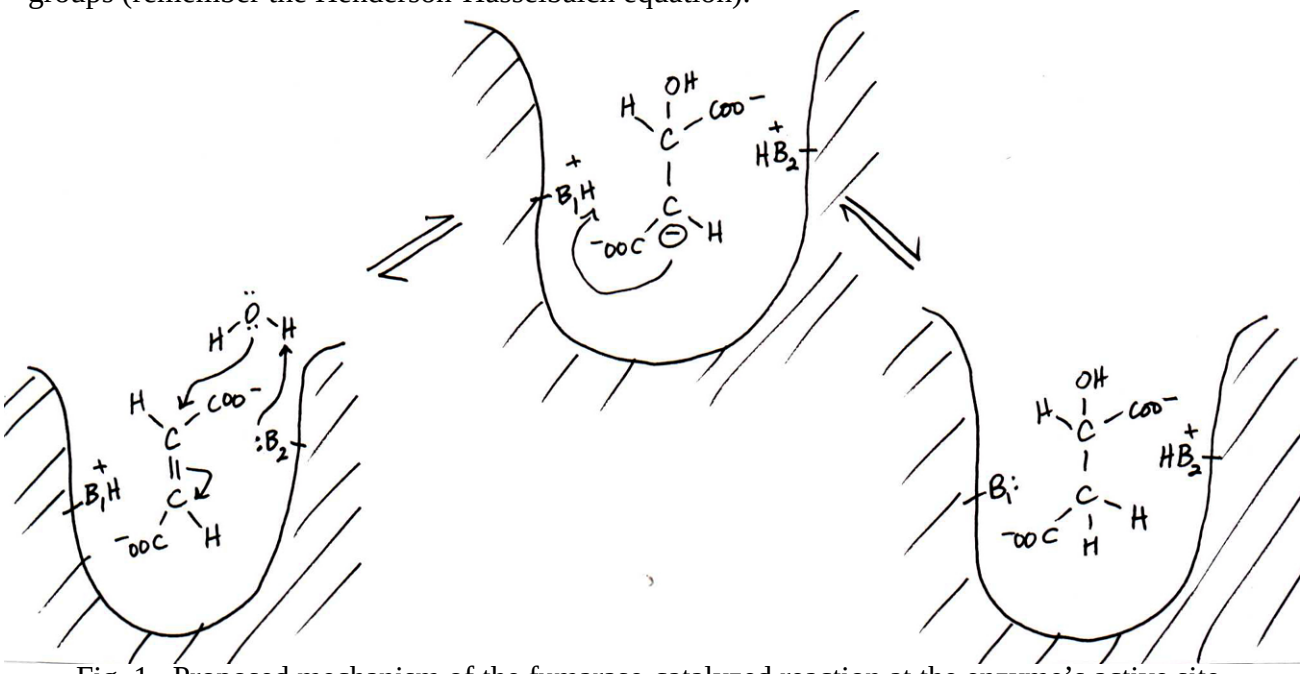


Fig. 1. Proposed mechanism of the fumarase-catalyzed reaction at the enzyme's active site.

EXPERIMENTAL PROCEDURE

Materials provided:

NaH_2PO_4-H_2O (solid); FW 137.99
Deionized water
pH meter, 2.0 M and 0.2 M NaOH, 2.0 M and 0.2 M HCl, pH standard solutions
porcine fumarase (Sigma F1757); [~ 0.05 mg/ml fumarase in 1 mM phosphate (pH 7)];
fumarate stock solution; [6 mM fumarate in 1 mM phosphate (pH 7)]

In this experiment, you are responsible for planning and preparing a series of phosphate solutions of the same concentration but with various pH values using sodium phosphate salt and water as your starting materials. In making your solutions, you will need sufficient volume to adjust the pH using a pH meter, but you should avoid making a huge excess. It is best to bring solutions to volume after adjusting the pH to help attain desired phosphate concentrations. Thus, it is advantageous to carefully plan your solution making. Once you have them made, you will use these solutions to spectrophotometrically assay the rate of the fumarase-catalyzed reaction as a function of pH. **Appendix II. Spectroscopy** and **Appendix III. Buffers** are recommended reading for lab preparation. (Note that phosphate does not buffer well at all pH'es.)

1. Prepare 18 mM sodium phosphate (NaH_2PO_4-H_2O) buffers over a seven pH unit range. *Think and plan carefully about how best to make this series of buffers. Feel free to check your calculations with the instructor before making the buffers*. You will need approximately 2 ml of each buffer in this experiment per group; *more* if you make mistakes. Consider coordinating buffer preparation with other groups (~2 buffers/group), so that you do not make excess buffer.

2. Using the provided 6 mM fumarate stock solution, prepare by dilution at least 2 ml of a 1 mM fumarate solution using each of your phosphate solutions, separately, as diluent.

3. Set the spectrophotometer to measure absorbance at 260 nm. Zero the absorbance with phosphate solution using a quartz cuvette (4 mm x 10 mm). Remove the solution, and carefully add 990 µl of fumarate solution to the cuvette. The fumarate in this solution should show a strong absorbance at 260 nm in the absence of enzyme. To determine the reaction rate;

a) remove the cuvette from its holder and add 10 µl of fumarase to the cuvette
b) quickly cover the cuvette with the white plastic lid and mix by inverting the cuvette twice
c) replace the cuvette and record the absorbance over time until the slope levels

The mixing should be done carefully and as rapidly as possible. Obtain two measurements of absorbance over time at each pH. Record the slope for the **initial linear range** of the reaction.

DATA ANALYSIS

After completing the laboratory, you are to turn in an analysis of your data (a table with *all* of the data (table of rates) and a plot of the pH dependence) plus brief answers to the questions below.

Using the slope value for the initial linear range of a spectrum, calculate the change in substrate concentration over time (i.e. Δ[fumarate]/min is the rate of the enzyme-catalyzed reaction) at each pH using the Beer's-Lambert law. Use the extinction coefficient of fumarate at 260 nm (ε_{260} = 900 $M^{-1}cm^{-1}$) and report your final answers in µmol fumarate/min (convert from ΔA/min). If you are unsure how to do this calculation, consult **Appendix II. Spectroscopy**. Plot the log of the rate of the reaction versus pH (pH is a log scale). Determine the optimal pH range for the enzyme.

1. What is the pH optimum for fumarase? What would be a good buffer for the fumarase catalyzed reaction (see Appendix III)? Explain.

2. Biochemists often mix mono- and di-sodium phosphate solutions to prepare phosphate buffers with a specific pH. What disadvantage does that approach have over the one you used for this experiment?

3. What are possible explanations for the pH dependence of fumarase based upon the enzyme's catalytic mechanism (see reference). Which amino acids may contribute acidic or basic groups to this reaction?

4. Is it possible for an acidic or basic amino acid side chain to function in an enzyme-catalyzed reaction at pH 7 if the side chain pK_a is far from 7? How would the native environment of the active site of the enzyme affect the pK_a of the acidic and basic groups involved in the reaction? (Consider the tendency of these groups to be protonated or deprotonated.)

5. Would you expect the pH optimum to be the same for the reverse reaction? Why or why not?

Reference: Weaver & Banaszak (1996) *Biochemistry* **35**, 13955-13965.

EXPERIMENT 5. Kinetic Analysis of Fumarase

From what you learned from Experiment 4, you can now analyze the effects of other environmental variables upon the catalytic activity of the citric acid cycle enzyme fumarase, while keeping constant the pH that promotes optimal enzyme activity. One way a biochemist can analyze the catalytic activity of an enzyme is through study of its reaction rates, or kinetics. The goal of this experiment is to analyze the reaction rates of fumarase in the forward and reverse direction as a function of changes in substrate concentration and as inhibited by citrate. Substrate and inhibitor concentrations are common variables of an enzyme's environment that influence its activity.

The simplest model for enzyme kinetics states that the formation of an enzyme-substrate (ES) complex is a necessary intermediate in catalysis, as proposed by Michaelis and Menten. When you think about it, if the formation of an ES complex is a necessary intermediate in catalysis, then the reaction rate (V_o) will depend on the formation of the ES complex. As formation of the ES complex will depend on the amount of substrate available, the enzyme activity (V_o) will vary with substrate concentration ([S]). For enzymes that fit the Michaelis-Menten model (including fumarase), this is described by the Michaelis-Menten equation:

$$V_o = \frac{V_{max}[S]}{K_M + [S]}$$

At a fixed enzyme concentration and low substrate concentrations, the rate of an enzyme catalyzed reaction increases linearly with increases in substrate concentration. However, when the active sites of an enzyme become saturated at high substrate concentrations, the reaction rate is independent of substrate concentration; the maximal velocity (V_{max}) is reached. The Michaelis-Menten constant (K_M) reflects the rates for formation and dissociation of the ES complex and formation of product, and is equal to the substrate concentration at half the value of V_{max}.

Remember that the fumarase-catalyzed interconversion of malate and fumarate can be followed by measuring the increase or decrease of near-UV absorption by the conjugated double bond of fumarate over time. Lacking a similar bond, malate does not absorb light in this range. By using this property of fumarate, the fumarase-catalyzed reaction can be analyzed kinetically in both the forward and reverse direction.

As you will be changing the fumarate concentration in the reaction for part of this analysis, there is the potential to achieve a corresponding increase in absorbance. It will be important to insure that you do not exceed the absorption range that can be accurately measured by the spectrophotometer. To accomplish this, you will make some of your measurements at wavelengths other than 260 nm. Measurements made at higher concentrations than 1 mM can be done using longer wavelengths, which have lower molar absorptivities, and visa versa. You will need to adjust your calculations for each wavelength by using the corresponding molar absorptivity of fumarate listed below.

Wavelength (nm)	ε ($M^{-1}cm^{-1}$)	Wavelength (nm)	ε ($M^{-1}cm^{-1}$)
220	9300	260	900
230	4520	270	535
240	2440	280	278
250	1450	290	118

A plot of $1/V_o$ verses 1/[S] (Lineweaver-Burke plot) allows you to determine the kinetic parameters V_{max} and K_M. See Chapter 8 in the Lehninger text for a refresher on kinetic analysis.

EXPERIMENTAL PROCEDURE

Materials provided:

assay buffer: [15 mM sodium phosphate (pH - *determined in Expt. 4*)]
malate stock solution [24 mM L-malate in phosphate buffer (pH 7.0)]
fumarate stock solution [6 mM in phosphate buffer (pH 7.0)]
citrate stock solution [100 mM in phosphate buffer (pH 7.0)]
porcine fumarase [~0.05 mg/ml in phosphate buffer (pH 7.0)]

1. **Dilutions:** You must make a series of dilutions of malate (to 8, 4, 2, 1, 0.5 and 0.25 mM in phosphate buffer) and fumarate (4, 2, 1, 0.5, 0.25, and 0.125 mM in phosphate buffer) in the absence and presence of 8 mM citrate. You will need about 1 ml of each dilution per assay. If you need to repeat the assay with one of the solutions, you will need to make more. *You may check calculations with the instructor before beginning.* You need not complete all dilutions before beginning step 2.

2. **Malate:** Review details of assay protocol in Expt. 4. Set the spectrophotometer to 240 nm wavelength. Carefully pipet 990 µl of diluted malate solution into a quartz cuvette. Place the cuvette in the spectrophotometer and zero the absorbance at 240 nm. Add 10 µL of fumarase, mix by inversion, place the cuvette in spectrophotometer and record the absorbance at 240 nm for several minutes. Find the slope of the initial linear portion of the time scan. Repeat for all of the diluted solutions of malate. Calculate the rate in µmol/min (vol = 1 mL) using ΔA/sec and the proper extinction coefficient (see above).

3. **Fumarate:** Set the spectrophotometer at one of the following wavelengths depending on the concentration of fumarate:

280nm/4 mM	250 nm/0.5 mM
270 nm/2 mM	240 nm/0.25 mM
260 nm/1 mM	230 nm/0.125 mM

Zero the spectrophotometer at the appropriate wavelength with buffer in the quartz cuvette. Remove the buffer, carefully pipet 990 µl of diluted fumarate solution into the cuvette, and place it in the spectrophotometer (the absorbance should be about 0.6-1). Add 10 µL of fumarase, mix as before, place the cuvette in the spectrophotometer and record the absorbance for several minutes. Find the slope of the initial linear portion of the time scan, and calculate the rate in µmol/min (vol = 1 ml) using ΔA/sec and the appropriate extinction coefficient (see above).

> **Note:** because you are using different wavelengths for each of these measurements, the change in rate as a function of fumarate concentration will not be reflected by the slopes you measure. This is because with each change of wavelength, there is a change in the corresponding molar absorptivity. You will see the changes in rate after your calculations are complete.

4. **Citrate:** Repeat steps 2 and 3 with substrate reaction mixtures containing 8 mM citrate in addition to varying concentrations of malate or fumarate. In each case, calculate the rate of the enzyme-catalyzed reaction in µmol/min using the appropriate extinction coefficients.

DATA ANALYSIS

Determination of K_M and V_{max}. Using the activity of the enzyme (rate = µmol/min) at various substrate concentrations (in mM) (without inhibitor), determine the kinetic parameters of the forward and reverse reactions catalyzed by fumarase. A plot of $1/V_o$ verses 1/[S] (Lineweaver-Burke plot) allows you to determine the kinetic parameters V_{max} and K_M from the line equation for the linear regression analysis of the data points. Be sure to use the correct units for both V_o and [S], or you will not obtain meaningful values for these parameters. Calculate the turnover number from the concentration of enzyme and the molecular weight of fumarase (MW = 194 kDa). For this calculation, you will need to have the enzyme rate reflected in M/min, which can be converted from µmol/min using the reaction volume (vol = 1 mL). You can also calculate the equilibrium constant for this reaction using the kinetic parameters obtained and the Haldane relationship:

$$K_{eq} = \frac{V_{max_{(fum)}} K_{M_{(mal)}}}{V_{max_{(mal)}} K_{M_{(fum)}}}$$

Determination of K_M' and V_{max}'. Repeat the same type analysis as above with the data obtained in the presence of inhibitor. The corresponding kinetic parameters obtained in the presence of inhibitor are indicated as K_M' and V_{max}'. Using the kinetic parameter that is most affected by the presence of inhibitor, determine the type of inhibition (competitive or noncompetitive) exhibited by citrate. Using this altered kinetic parameter and one of the equations below, calculate the inhibitor constant (K_i) for citrate in the forward and reverse reactions.

$$V'_{max} = \frac{V_{max}}{(1 + \frac{[I]}{K_i})} \qquad \textbf{OR} \qquad K'_M = K_M(1 + \frac{[I]}{K_i})$$

For Expt. 5 you are to turn in at the end of the laboratory an analysis of your data (graphs of $1/V_o$ verses 1/[S] and a table of kinetic parameters; K_M, V_{max}, K_M', V_{max}', and K_i) plus brief answers to the questions below.

1. How do the kinetic parameters you obtained for porcine fumarase compare to those reported in the literature (Alberty *et al*., 1954, Table V on reserve)?

2. Which substrate is preferred by the enzyme? How do you know?

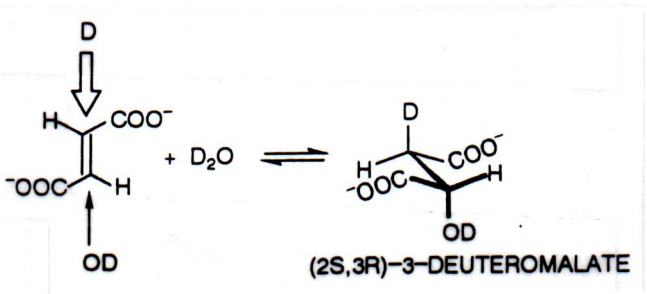
3. What type of inhibitor is citrate in the fumarase-catalyzed reaction? How do you know? Suggest a mechanism of inhibition by citrate.

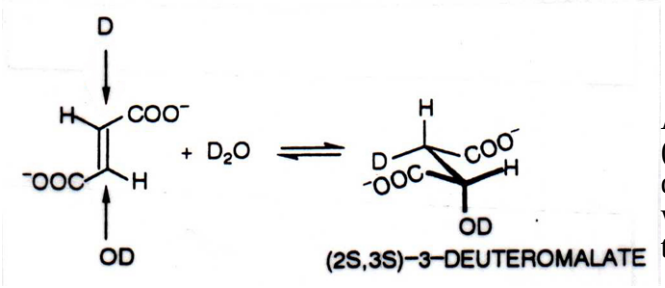
4. Is citrate an equally effective inhibitor in the forward and reverse reactions? How do you know?

Reference: Alberty et al. (1954) *J. Amer. Chem. Soc.* **76**, 2485-2493.

EXPERIMENT 6. Stereochemistry of the Fumarase Reaction — an NMR Study

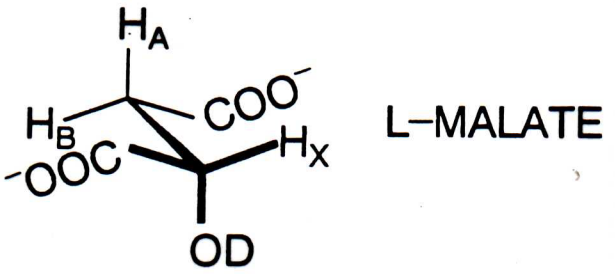
As foreshadowed in Experiment 4, the reaction catalyzed by fumarase is stereospecific. In aqueous solutions, it is not possible to determine the stereochemistry of the addition of H_2O across the double bond of fumarate since there is no way to determine which of the two hydrogen atoms was added to carbon-3 of malate from water. However, the use of deuterium oxide (D_2O) as the solvent makes it possible to follow the stereochemistry. For example, if the reaction involves an *anti* (or *trans*) addition of water, (2S,3R)-3-deutereomalate is produced (right).





Alternatively, if the reaction involves an *syn* (or *cis*) addition of water, (2S,3S)-3-deutereomalate is produced (figure left). Either way, two new chiral centers are produced by the stereospecific addition of D_2O to fumarate.

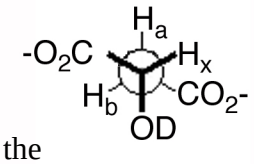
The stereochemistry of the fumarase reaction can be demonstrated by NMR spectroscopy. The NMR spectrum of fumarate is quite simple. It consists of a singlet at δ6.5 from the two equivalent hydrogens in addition to an HOD peak at about δ4.7 (due to HOD in the solvent). The spectrum of L(S)-malate is more complex, but still easily interpreted.



Malate contains three different protons (H_A, H_B, and H_X). Note that protons A and B are not equivalent. This is a general result for protons adjacent to an asymmetric center. These two protons are *diastereotopic*, in that the independent substitution of either of these protons by any other group creates a diastereomer (a stereoisomer that is not a mirror image). Since these two protons have different magnetic environments, they have different chemical shifts and couple with one another (*geminal* coupling, since both protons are bonded to the same carbon). Coupling between HA or H_B and H_X is called *vicinal* coupling, since the two protons are attached to adjacent carbons. Thus, each proton is split by each of the other two protons, making the resonances for each a doublet of doublets. For example, H_A is split by H_B and the lines that result are each split by H_X.

When the fumarase enzyme is added to a solution of L-malate in D_2O, the fumarate peak at δ6.5 grows while the malate peaks decrease in intensity. The malate spectrum also begins to *simplify* (although is may not *look* simpler at first) due to new malate being formed as equilibration of the reaction proceeds. Proton NMR does not "see" deuterium, so one set of malate peaks disappears as a proton is replaced by deuterium. Deuterium does not couple with protons, so for each of the remaining doublet-of-doublets, only one of the couplings will remain.

By knowing the expected sizes of coupling constants for vicinal protons at various dihedral angles (described by the Karplus curve), one can determine which of the protons (H_A or H_B) was replaced by deuterium. This in turn allows determination of whether the D_2O addition is *syn* or *anti*.



The dihedral angle is the angle between H_x and H_a or H_b when looking down the bond between the two central carbon atoms of malate.

EXPERIMENTAL PROCEDURE

Materials provided:

malate solution [200 mM L-malate in 100 mM phosphate buffered D_2O (pH 7.3)]
fumarate solution [200 mM fumarate in 100 mM phosphate buffered D_2O (pH 7.3)]
porcine fumarase [1.0 mg/ml in 100 mM phosphate buffered D_2O (pH 7.3)]

Please read **Appendix V. NMR** before starting this experiment.

1. Place 0.8 ml of L-malate and 0.8 ml of fumarate in separate, clean, dry NMR tubes. Record the NMR spectrum of each.

2. Place 0.8 ml of L-malate and 0.8 ml of fumarate in microfuge tubes. Add 5 µl of fumarase to each tube and mix gently. Thirty minutes after addition of enzyme, transfer each volume to separate, clean, dry NMR tubes and record a spectrum of each.

DATA ANALYSIS

For Expt. 6 you are to turn in at the end of the laboratory an analysis of your data (a table of coupling constants (J) and all NMR spectra with labeled peaks, including chemical shift positions (δ)) plus brief answers to the questions below.

Assign each peak in the NMR spectra of L-malate and fumarate to protons in each molecule. Calculate the vicinal spin-spin coupling constants J_{AX} and J_{BX}, and the geminal constant J_{AB}. Identify and interpret the changes in the NMR spectra brought about by the enzyme-catalyzed reaction. Assign all the peaks in the final spectra (after enzyme addition).

1. What accounts for the difference in value of J_{AX} and J_{BX} in the spectrum of L-malate alone? Refer to the Karplus curve (see **Appendix V. NMR**) in your answer.

2. What changes in the NMR spectra after enzyme addition would you expect if the reaction was *syn*, *anti*, or non-stereospecific?

3. What is the stereochemistry of D_2O addition to fumarate?

References: Kasparek & Pratt (1977) *J.Chem.Ed.* **54**, 515.

Nigh (1976) *J. Chem. Ed.* **53**, 668.

Silverstein et al., (1974) in *Spectrometric Identification of Organic Compounds* (3rd edition, Wiley), pp. 181-182, 186-187, 189-191.

Teipel et al. (1968) *J. Biol. Chem.* **243**, 5684.

APPENDIX I. Concentration and Dilution

The most common expressions of concentration in biochemistry are *molarity* and *percent-of-solute*. **Molarity** is defined as the number of moles of solute per liter of solution (denoted M). Molarity usually describes solutions of accurate concentration, where the molecular weight of the solute is known. Solutes are weighed on an analytical balance, and volumes are measured in volumetric flasks. In biochemistry, most concentrations are in the millimolar (mM), micromolar (µM), or nanomolar (nM) range.

Percent-of-solute is frequently used for liquids and solids of undetermined molecular weight. Three designations are used: volume per volume (v/v), weight per volume (w/v), and weight per weight (w/w). A 5% (v/v) solution contains 5 ml solute in 100 ml total solution. A 5% (w/v) solution contains 5 g solute per 100 ml total solution. A 5% (w/w) solution contains 5 g solute per 100 g total solution. Of these three designations, weight per volume expressions are used most commonly in biochemistry. Sometimes these concentrations are not expressed as percentages, but rather simply as the number of mg (or µg) per ml solution.

Biochemical experiments frequently require the dilution of a particular sample or **stock** (standard) **solution**. If the concentration of stock solution and that needed in the experiment are known, the *dilution factor* can be calculated. For example, if you want to dilute a 1 M stock solution to 5 mM in your experiment, the dilution factor is 0.005/1 or 1/200 (also indicated as a 1:200 dilution). This means you must dilute 1 part stock solution with 199 parts solvent. Another way of stating this is that 1 part stock solution must be diluted into 200 parts total solution. This is commonly described by biochemists as a one ***in*** 200 (or two hundred-fold) dilution. However, this would be a mixture with a ratio of one (part) to 199 (parts). Generally, if you want 200 ml of the diluted solution, you would mix 1 ml stock solution with 199 ml solvent. However, this neglects any volume change that may be inherent to formation of the new mixture. A more rigorous approach would be to add 1 ml stock solution to a volume of solvent less than the total desired, and then bring the solution to the final desired volume with additional solvent.

You can determine the molarity of a diluted solution (M_{final}) or calculate what volume of stock solution ($V_{initial}$) must be added to solvent to make the diluted solution by using the formula:

$$M_{initial}V_{initial} = M_{final}V_{final}$$

For example, if you have a stock solution of 10 M NaCl, and you want to make 250 ml of a 0.5 M NaCl solution, you need to add 12.5 ml of the stock solution to a 250 ml volumetric flask and fill it with solvent to 250 ml.

$M_{initial}$ = 10 M $V_{initial}$ = ? M_{final} = 0.5 M V_{final} = 250 ml

$$(10\ M)V_{initial} = (0.5\ M)(250\ ml) \text{ --------> } V_{initial} = 12.5\ ml$$

In other words, 12.5 ml of the stock solution is diluted to 250 ml with solvent. Note that the number of moles of solute (in this case, NaCl) is the same in the volumes of the initial and final solutions. Note also that this equation can be written $C_{initial}V_{initial} = C_{final}V_{final}$, where C represents concentration in units other than molar (e.g., mg/ml).

A special type of dilution is the *serial dilution*. In this method, a stock solution is diluted systematically in fixed steps of the same dilution factor (see Fig. 1). A high degree of accuracy and precision is required, because errors made in an early dilution are carried to all subsequent dilutions. Serial dilutions are used frequently in immunological and microbiological experiments.

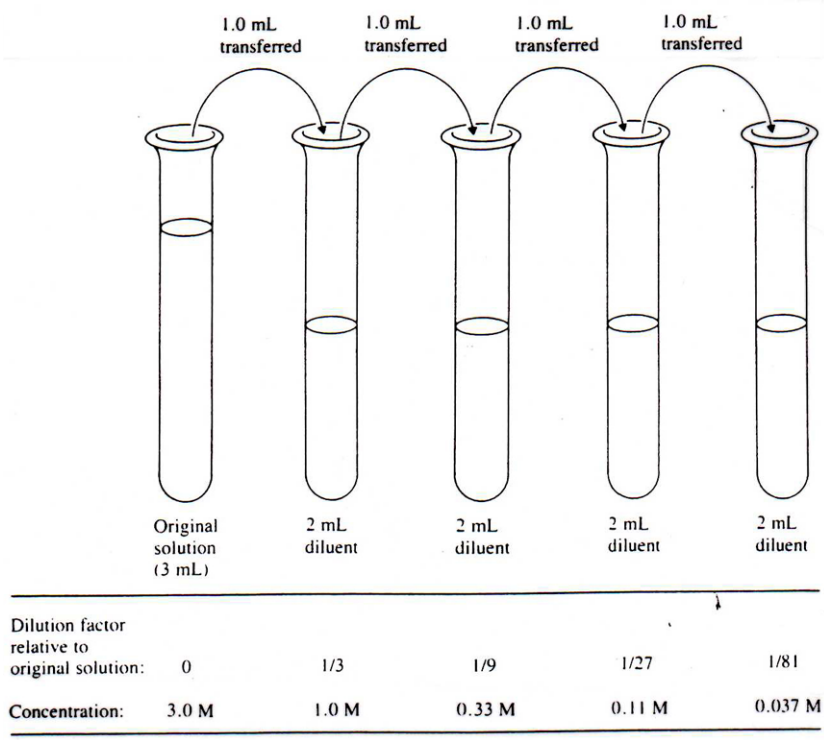


Fig. 1. A serial dilution scheme.

APPENDIX II. Spectroscopy

1. Absorption Spectroscopy

If white light is passed through a colored solution, certain wavelengths of light are selectively absorbed, and the resulting color observed is due to the wavelengths of the transmitted light. UV/Vis absorption spectroscopy is useful if the sample absorbs light in the 200-350 nm range (ultraviolet, UV) or in the 350-750 nm range (visible). The figure below (Fig. 1) shows the absorption spectrum of riboflavin. Riboflavin appears yellow to the eye. Maximal absorption of light occurs at several wavelengths in the ultraviolet region (below about 370 nm), and at 450 nm in the visible region. Since the absorption in the visible region is at the blue end, the transmitted light is that of the other end of the spectrum (yellow).

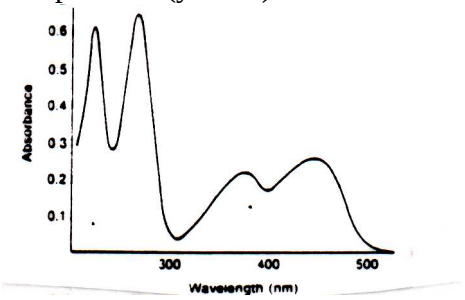


Fig. 1. Absorption spectrum of Riboflavin

The quantitative measurement of the amount of light absorbed is based on two laws. Lambert's law states that the proportion of light absorbed is independent of its intensity, and each successive portion of the medium absorbs an equal fraction of the light passing through it. This is expressed mathematically as:

$$\log (I/I_o)=Kl$$

where I_o and I are the intensities of the incident and transmitted light, respectively, K is a constant, and l is the path length in cm. Log (I/I_o) is the absorbance (A). This law is always true. The second law applied here, Beer's law, has some exceptions, usually because of a change in the state of the solute upon absorbing light. This could involve changes in ionization or aggregation. Beer's law relates concentration to the factor K. $K=\varepsilon c$, where ε is the extinction coefficient of the absorbing solute (units are reciprocal concentration times reciprocal length) and c is its concentration. Combining these two laws gives the Beer's-Lambert law:

$$A=\varepsilon cl$$

Absorption is dimensionless. If c has units of M, ε has units of $M^{-1}\ cm^{-1}$; if c has units of mg/ml, ε has units of ml $mg^{-1}\ cm^{-1}$. The extinction coefficient reflects the relative tendency of a solute to absorb light of a certain frequency, and is specific only for a specific solute, solvent, and wavelength of light.

UV/Vis absorption spectroscopy can be used to quantitatively measure enzyme activity if a substrate or product of the enzyme-catalyzed reaction absorbs light. This type of assay follows the activity of the enzyme indirectly by monitoring the change in substrate or product directly. A good example of this is the redox coenzyme NAD^+/NADH, which changes its absorption properties upon oxidation and reduction (Fig. 2). The different absorption of light by NAD+ and NADH can be utilized to follow the activity of an enzyme involved in their interconversion.

An analysis of the spectra of both (Fig. 2) suggests that their absorptions differ most at ~340 nm wavelength light. This wavelength therefore provides the largest "window" to study their interconversion. By following the change in absorption at 340 nm over time as a function of enzyme activity, one can extract quantitative rate data using the Beer's-Lambert law and the extinction coefficient for NADH.

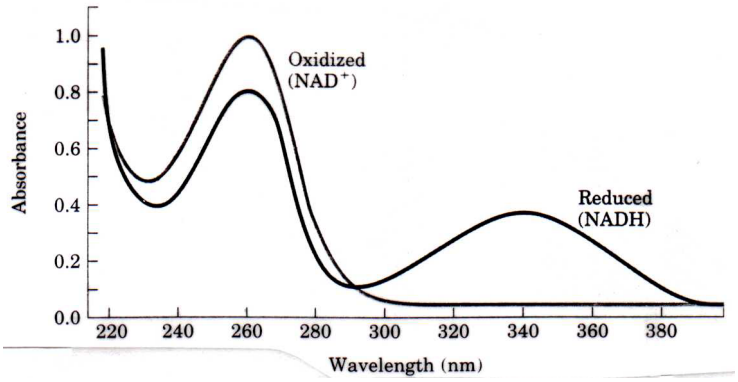


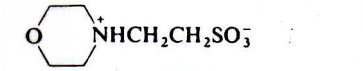
Fig. 2. Absorption spectra of NAD^+ and NADH

APPENDIX III. Buffers

Most cellular reactions and processes take place in an aqueous environment at neutral pH. Therefore, most biochemical experiments are also carried out in aqueous media under controlled pH conditions. This requires the use of **buffers**, solutions that resist changes in pH due to the presence of a chemical species that can "neutralize" added acid or base. In general, buffers are solutions of a weak acid and its conjugate base or a weak base and its conjugate acid. When acid is added to the buffer, some A^- is converted to HA; addition of a base results in some HA converting to the A^- form. Thus the solution can "absorb" added acidic and basic species.

A buffer is effective for use in a particular biochemical experiment based upon its 1) pK_a value and chemical properties and 2) concentration. The pK_a indicates if a buffer can function well over the an appropriate pH range for the experiment. The concentration indicates the capacity for which a buffer can function. The Henderson-Hasselbalch equation [$\mathbf{pH = pK_a + \log ([A^-]/[HA])}$] gives the relationship between the pH of a solution, the pK_a of an acid in solution, and the ratio of the concentrations of acid [HA] and conjugate base [A^-] forms in solution.

Buffering range:
When the concentrations of A^- and HA are equal, the pH of the solution equals the pK_a of the buffer. Buffers are most effective in the range pH = $pK_a \pm 1$. Outside of this range, the concentration of acid or conjugate base is too small to "neutralize" acid or base added to the solution. Thus, once the desired pH range has been determined for an experiment, a buffer can be selected based upon its pK_a value. The pK_a values for certain useful buffers is shown below.

Buffer	pK_a (20°C)	Structure
Phosphoric acid	2.1	H_3PO_4
Sodium dihydrogen phosphate	7.2	NaH_2PO_4
Disodium hydrogen phosphate	12.3	Na_2HPO_4
Citric acid	3.1	$HOOCCH_2COH(COOH)CH_2COOH$
Sodium dihydrogen citrate	4.7	$HOOCCH_2COH(COONa)CH_2COOH$
Disodium hydrogen citrate	5.4	$HOOCCH_2COH(COONa)CH_2COONa$
Glycylglycine	8.4	$H_3N^+CH_2CONHCH_2COO^-$
Tris [tris(hydroxymethyl)-aminomethane]	8.3[a]	$(HOCH_2)_3CNH_2$
MOPS [3-(*N*-morpholino) propanesulfonic acid]	7.2	
MES [2-(*N*-morpholino) ethanesulfonic acid]	6.2	
HEPES [*N*-2-hydroxyethyl-piperazine-*N'*-2-ethane-sulfonic acid]	7.6	

[a]The pH of a Tris buffer varies significantly with temperature ($\Delta pK_a = -0.31$/°C).

Table 1. Properties of some common biological buffers.

Buffering capacity:
The concentration of buffer used is also important, in order to provide adequate *buffering capacity*. Buffering capacity depends upon the concentration of the buffering components, and is defined as the number of equivalents (n) of either H^+ or OH^- required to change the pH of a given volume of buffer by one pH unit.

$$\text{Buffer capacity} = d\text{n}/d(\text{pH})$$

Most biological buffers are made in the concentration range of 0.01-0.5 M; this concentration is referred to as the *buffer strength* and reflects the total concentration of acid and conjugate base forms of the buffer. Buffer solutions can be prepared by calculating the amount of acid and conjugate base forms of the buffer needed to obtain the desired pH, using the Henderson-Hasselbalch equation. Another method is to weigh out the desired amount of one form of the buffer (HA or A^-) and adjust the pH using a pH meter and solutions of NaOH, HCl, or Tris.

The chemical properties of a buffer (e.g., solubility, stability, light absorption, ionic interactions) must be well-characterized before use in a biochemical experiment. A series of biologically useful buffers for use in the pH 7-9 range (MOPS, HEPES, MES, PIPES, etc.) have been characterized by Good et al. (1966). Note that these buffers are not physiological (i.e., not found in the body).

Reference: Good et al. (1966) *Biochemistry* **5**, 467

APPENDIX IV. Enzymology

Enzyme assay. The activity of an enzyme can be determined by measuring the rate of utilization of substrate over time or the rate of formation of product over time. Enzyme activity is reported as Δ[substrate]/Δtime or Δ[product]/Δtime (also denoted *units* of enzyme activity). The unit definition for enzymes varies, but is often expressed as µmoles substrate used or product formed per min in the assay mixture at a specified temperature and pH.

One unit of enzyme activity = One µmol S (or P)/min

Enzyme assays are carried out under buffered conditions at a fixed pH, temperature, and concentration of necessary ions, and in the presence of excess substrate (usually at least [S] = 10 x Km). Appropriate negative controls should be done in the absence of enzyme and/or substrate. Changes in the measured parameter in the absence of enzyme indicates the extent of non-enzymatic reaction in the assay mixture. Changes in the measured parameter in the absence of substrate indicates the extent of background enzymatic reaction in the enzyme preparation. Enzyme activity can be measured by a *continuous (kinetic)* method or a *discontinuous (fixed-time)* method; a continuous assay is preferred.

$$V_o = \frac{k_{cat}[E_{tot}][S]}{K_M + [S]}$$

In purifying an enzyme, one must determine the amount of enzyme present at each step of the purification. To do this, enzyme activity in various purification fractions is measured under conditions of fixed substrate concentration, pH, temperature, and added ions, coenzymes, etc. The Michaelis-Menten equation (shown below) relates the initial velocity, V_o, to the total amount of enzyme, E_T, at a fixed substrate concentration.
Since the initial velocity is directly proportional to the total amount of enzyme, careful measurement of the initial velocity under these fixed conditions gives a measure of the amount of enzyme.

A major problem in developing an enzyme assay is establishing conditions that allow accurate measurement of the initial velocity. This problem is minimized if high [S] is used, so that [S] doesn't change appreciably during the assay. The amount of enzyme used in an assay is also important. Too little enzyme gives a velocity too low for accurate measurement, while too much enzyme gives a velocity too high and not linear long enough to determine an accurate initial rate. The process by which the optimal amount of enzyme to be used in an assay is determined is called *range-finding*. A plot of initial velocity *vs.* amount of enzyme added should have a linear portion that defines the valid range of [E] for this assay.

Enzyme purity. When purifying an enzyme, one needs to determine the yield and degree of purity of the enzyme as the purification progresses. Yield is measured by comparing the *total enzyme activity* (units) in various enzyme fractions. Purity is measured by comparing the *specific activity* (units/mg protein) in various fractions. The higher the specific activity, the more pure the enzyme. Purity is usually expressed as a *purification factor* (or *fold-purity*), which is the increase in specific activity. The effectiveness of a purification scheme is shown in a *purification table* (Table 1), which lists the total and specific activities for the various fractions generated during the purification.

Enzyme fraction	Total Act. (Units)	Specific Act. (Units/mg)	Yield	Purity
Crude cell extract	1,030	1.2	100%	---
Ion exchange column	807	13.5	78%	11-fold
Affinity column	498	244	48%	203-fold

Table 1. Sample purification table.

Dialysis. Dialysis is a technique commonly used in biochemistry to separate a mixture of dissolved solutes by filtration (Fig. 1). A mixture is placed within a sealed dialysis membrane and then submerged in buffer, and small solute molecules that are not highly charged pass freely across the membrane until a concentration equilibrium on both sides of the membrane is achieved.

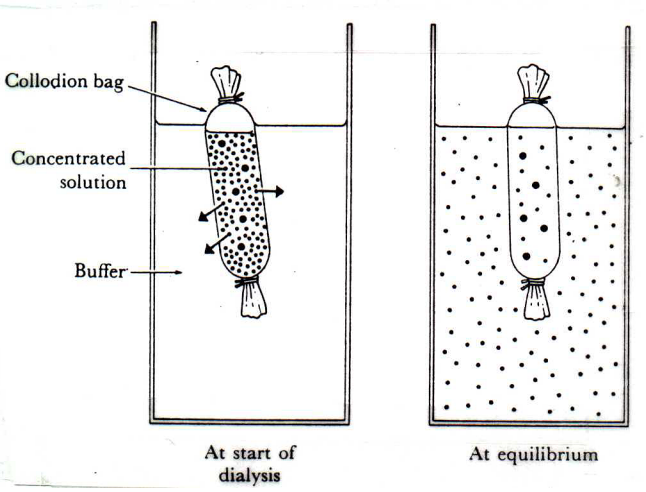
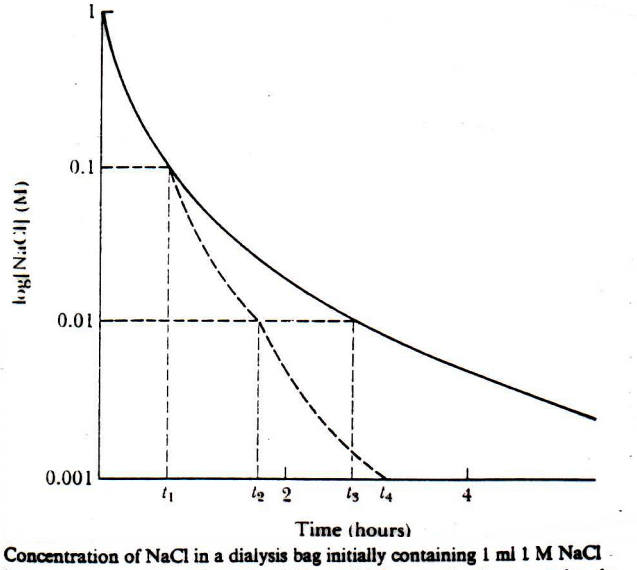


Fig. 1. Dialysis of a mixture of solutes

The driving force for solute movement is the initial *concentration gradient* across the dialysis membrane. Dialysis is often used to remove salts or other small solutes from a protein sample during the course of a purification. The concentrations of solutes within the dialysis membrane change because water passes freely across the membrane in the direction of higher initial solute concentration (the *osmotic effect*).



Concentration of NaCl in a dialysis bag initially containing 1 ml 1 M NaCl immersed in water, as a function of time (solid line). At the times t_1 and t_3 the concentration has dropped tenfold and 100-fold, re- ectively. The dashed line shows the result of transferring the bag to fresh water at time t_1, after which the 100-fold decrease is reached at time t_2, which is less than t_3. At t_2 there is a second transfer to fresh water resulting in completion of a 1000-fold total dilution by t_4; without these two transfers it would have taken infinite time to achieve this dilution.

Fig. 2. Dilution of NaCl by dialysis

The dilution factor of the small solutes can be calculated by dividing the initial volume of sample by the amount of buffer that the sample is dialyzed against. Equilibration usually takes several hours, so samples are often left to dialyze overnight at 4°C. Large dilutions can be obtained in shorter times by changing the buffer at least once during the dialysis. In this case, the dilution factors due to each volume of buffer used are multiplied by each other to give the final dilution. For efficient dialysis the buffer in the outer reservoir should be stirred. Fig. 2 shows the dilution of NaCl over time due to dialysis.

APPENDIX V: Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) is a very useful technique to study the structure of many types of biomolecules. NMR is useful when biomolecules contain inherently magnetic nuclei (^{1}H, ^{2}H, ^{13}C, ^{14}N, ^{19}F, ^{31}P); ^{1}H and ^{13}C NMR are used most frequently.

NMR is based on the behavior of (+)-charged spinning nuclei in an externally applied magnetic field (H_0). In the absence of H_0, the axes about which the nuclei spin are oriented randomly in solution. When H_0 is applied, the nuclei align parallel or antiparallel to the external field. The parallel orientation is slightly lower in energy and thus slightly favored. Irradiation of aligned nuclei with radiation of a certain frequency causes nuclei to absorb energy and *spin-flip* to a higher energy state (and in doing so, be in *resonance* with the applied radiation).

All nuclei in molecules are shielded somewhat from H_0 by surrounding electrons, which set up tiny local magnetic fields. So the effective magnetic field (H_{eff}) experienced by each nuclei is lessened by these local magnetic fields (H_{local}).

$$H_{eff} = H_0 - H_{local}$$

Most nuclei in a molecule are in a slightly different electronic environment, so H_{eff} differs for each.

NMR spectra show the intensity of absorption *vs*. **chemical shift** (an indication of H_{eff}). Each nonequivalent nucleus will have its own characteristic peak on the spectrum. Two protons bonded to the same carbon are not necessarily equivalent. In the case of L-malate (Fig. 1), protons A and B are *diastereotopic*, because the substitution of either by any other chemical group creates diastereomers (stereoisomers that are not mirror images) due to the presence of another stereogenic center in the molecule. Thus H_A and H_B have their own characteristic peaks on an NMR spectrum.

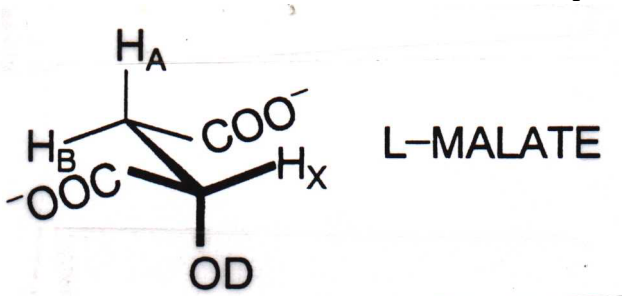


Fig. 1. Structure of L-malate.

NMR spectra are calibrated by using a reference sample, usually tetramethylsilane, TMS [$(CH_3)_4Si$]. The chemical shift of TMS is set at zero; samples absorb *downfield* (to the left of the standard on the chart). The unit of chemical shift is the delta unit, δ.

$$1\ \delta = 1\ \text{ppm of the operating frequency of the NMR spectrometer}$$

Gustavus College has a 300 MHz machine, so 1 δ = 300 Hz. Most 1H NMR absorptions occur 0-10 δ downfield of the reference peak. For example, an *allylic proton* (=C-C-H) absorbs at δ = 1.5-2.5, and a *vinylic proton* (C=C-H) absorbs at δ=4.5-6.5.

The nuclear spins of adjacent atoms can influence each other, causing **spin-spin coupling** or **splitting**. In ^{1}H NMR, splitting occurs between protons coupled via covalent bonds and usually no more than two carbons apart. A general rule when studying spectra is that the peaks of protons with *n* equivalent neighboring protons split into a multiplet of *n+1* peaks. The distance between peaks in a multiplet is the *coupling constant*, **J**. In general, two groups of protons coupled to each other have the same value of J. Stronger coupling is indicated by greater spacing of the split peaks. *No splitting is observed if adjacent protons are chemically equivalent.*

For *vicinal* protons (those on adjacent carbons), the value of J is related to the dihedral angle, ϕ; this relationship is shown in the *Karplus curve* (Fig. 2). The coupling of *trans* protons is greater than that between *cis* protons. In this case, NMR is useful to distinguish between *cis-trans* isomers. Dihedral angles of 60° and 180° yield J values of 2-4 and 8-10, respectively.

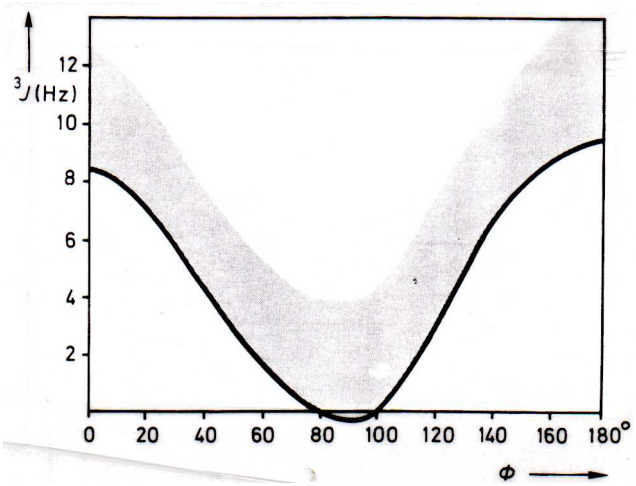


Fig. 2. Karplus curve. Line, theoretical curve; shaded area, range of empirical results

APPENDIX VI. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel electrophoresis is an effective method for separating proteins on the basis of size. SDS-PAGE is a electrophoretic technique that uses a gel made of polymerized acrylamide. Acrylamide, CH_2=CH-C(=O)-NH_2, forms a linear polymer, polyacrylamide, that can be cross-linked with *N,N'*-methylene bisacrylamide, CH_2=CH-C(=O)-NH-CH_2-NH-C(=O)-CH=CH_2, to form a resin of controlled but varying pore size. Cross-linked polyacrylamide is shown in Fig. 1.

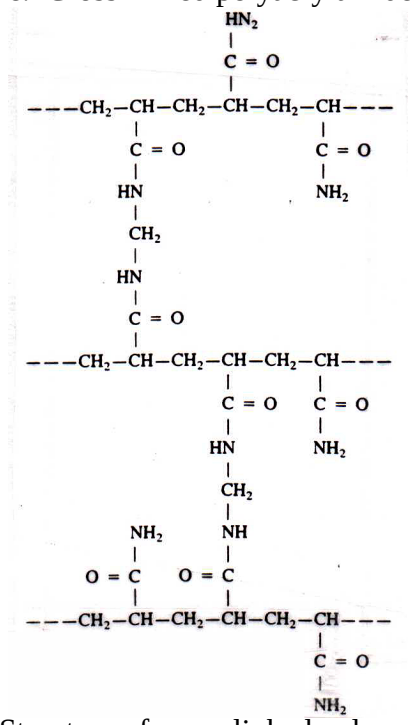


Fig. 1. Structure of cross-linked polyacrylamide

Polymerization is catalyzed by free radicals, generated by agents such as ammonium persulfate in the presence of *N,N,N',N'*-tetramethylethylenediamine (TEMED). Polymerization is inhibited by oxygen, so gel solutions should be degassed. If the pore size of the gel approximately matches the size of the molecules to be separated, smaller molecules can move freely in an applied electric field whereas larger molecules have restricted movement.

SDS-PAGE most often makes use of vertical slab gels, although gels can also be formed in cylinders. A discontinuous gel system is also used frequently, consisting of a stacking gel and a separating gel. The stacking gel makes up only approx. 10% of the volume of the total gel and is of a lower percentage acrylamide (usually 2.5-4.5%) and a lower pH (6.8). In the stacking gel, charged molecules move freely in an applied electric field and proteins in a sample should accumulate in *stacks* of closely spaced bands. In the separating gel, containing a higher percentage acrylamide (usually 7-15%) and at a higher pH (8.8), proteins separate into discrete bands based on molecular size. See Fig. 2 for sieving properties of different percentage gels.

Percentage acrylamide in separating gel	Size of molecules sieved effectively
2.5%	10^5-10^6 daltons
7.0%	10^4-10^5 daltons
30%	10^2-10^3 daltons

Fig. 2. Molecular sieving properties of gels of varying polyacrylamide percentages.

Separation is not on the basis of shape or charge, because proteins are treated with sodium dodecyl sulfate (SDS), a detergent with both polar and nonpolar properties. The long hydrocarbon chain of SDS is buried in nonpolar regions of proteins and the (-)-charged sulfate is exposed on the surface of proteins. SDS coats proteins with an approximately uniform charge-to-mass ratio of (-) charge (saturation occurs at approx. 1.4 g SDS/g protein). In addition, proteins are approx. uniformly shaped into spheres when coated with SDS, because the detergent disrupts native ionic and hydrophobic interactions. Thus, proteins separated by SDS-PAGE are denatured. Disulfides in proteins are broken by addition to the gel sample buffer of ß-mercaptoethanol or 1,4-dithiothreitol, both of which reduce disulfide bonds.

The molecular weights of proteins in a sample can be estimated on a gel by measuring the mobility of protein standards of known molecular weight on the same gel. A calibration curve (Fig. 3) can be generated from the mobilities of the standards by plotting the distances migrated from the top of the resolving gel versus the log of molecular weight. A linear regression can be calculated and used to estimate unknown molecular weights. The estimation of molecular weight by this method is accurate to approximately 5-10% of the actual value.

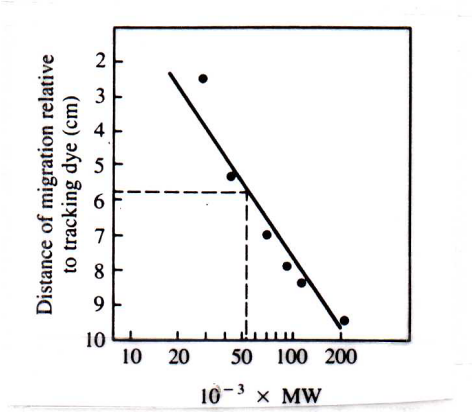


Fig. 3. Calibration curve for molecular weight estimation

Reference: U.K. Laemmli (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, p. 680.